Distr.
LIMITED

IPCT.93
16 November 1989

ORIGINAL: ENGLISH

UNITED NATIONS
INDUSTRIAL DEVELOPMENT ORGANIZATION

ENZYME ENGINEERING AT THE INDUSTRIAL LEVEL: PRESENT STATUS AND FUTURE PROSPECTS*

prepared by

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INTRODUCTION

1. Enzyme engineering is the new field of science and technology dealing with the development of useful products or processes based on the catalytic action of enzymes isolated from their source of synthesis or intact within cells that are immobilized and usually not growing. This definition (1) omits fermentation and tissue culture systems, in which living cells are growing, but does not omit multi-enzymes in sub-cellular particles.

2. From this definition it is apparent that enzyme engineering is an applied area of research and development. However, it may not be apparent that enzyme engineering has such a strong dependence on the results of fundamental research in chemical enzymology, physical chemistry, microbiology, biochemistry, and polymer chemistry and that often it is not possible to see a clear distinction between enzyme engineering and these fundamental sciences. For example, the immobilization of enzymes, the chemical modification of enzymes, studies into the mechanism of specific enzymes, and genetic manipulations to improve enzyme availability or process characteristics all are fundamental scientific topics which become part of enzyme engineering only when considered in the light of a specific enzyme-based process or product. Similarly, the fundamental topics of heat and mass transfer, diffusional effects on enzyme-catalyzed reaction rates, and mathematical modelling of enzyme-catalyzed reaction kinetics or overall process economics become part of enzyme engineering primarily when applied to a specific enzyme-based process or product.

3. In the final analysis an enzyme-based process or product must provide something unique if it is to find practical acceptance. Examples of suitable unique contributions include:

   (i) when it produces a new and useful product or carries out a new and useful chemical transformation or an old transformation in a new environment, e.g. in vivo,

   (ii) when it produces a better quality product, and
   (iii) produces an existing product more cheaply.

4. Traditionally, enzymes have been extracted from plants and animals. Rennin, for instance, the enzyme used to curdle milk to make cheese, is extracted from the stomach of unweaned calves. But the production of enzyme from bacteria, yeasts and fungi is rapidly becoming more common. At a rough estimate more than 50 enzymes are used only in the food industry throughout the world: in brewing, cheesemaking, the production of fruit juices and wines, starch and sugar processing and as meat tenderizers (2). Currently, just 16 enzymes of the thousands produced in nature rank as industrial catalysts, accounting for over 90% of the total market (3). Three enzymes, for instance, form the basis for an industry which manufactures liquid sweeteners from corniest (see below).

5. The use of enzymes as catalysts is growing remarkably. Frost & Sullivan reported recently that consumption of industrial enzymes in Western Europe will approximately double over 10 years in real terms from projected 1986 sales of about $ 160 million (3). In 1983 enzymes sales worldwide were reported to be about $ 390 million, up nearly 25 percent from 1979 (4), and in 1985, as reported in (5) the sales reached $ 500 million in the USA and Western Europe alone. A 1982 report from the Office of Technology Assessment predicted that, within 20 years, enzymes would be used in the production of chemicals and pharmaceuticals worth $ 15 billion. On a dollar basis, about 80 percent of enzymes are attributed to the food industry (6). On a volume basis, about 40 percent of sales are accounted for by starch conversion enzymes and another 30 percent by proteolytic enzymes used in laundry detergents. Europe dominates the world in sales, with Novo Industries in Denmark and Gist Brocades in the Netherlands controlling 60 per cent of the world market (2).

6. Immobilized enzymes (a similar definition may be given in relation to immobilized cells) may be defined as an enzyme whose free movement has been restricted in some manner. One of the major advantages of immobilization is to "fix" the enzyme so as to retain it in a continuous process. Use of soluble enzymes in processing has been limited in part by the cost of the enzymes due to the difficulty and expense of their isolation, their instability, and the fact that in a freely soluble form they usually can be used only once.

7. There are several advantages connected with the use of immobilized enzymes. An immobilized enzyme, in solid or sometimes semi-solid form, is readily separable from the product solution and can be reused, thus increasing productivity by a large factor. Additionally, it is possible to terminate the enzymic reaction easily by simply removing the enzyme. This gives more precise control of the reaction. Finally, enzymes (or cells) are often sufficiently more stable when in fixed form thereby allowing an enzymatic conversion to run for a much longer time without replenishing the biocatalyst compared with intact enzymes or cells. Specific examples will be given in the following sections of this review.

8. Enzymes (or cells) may be immobilized by several techniques.

These include:

(1) Physical adsorption;
(2) Covalent attachment to an inert support;
(3) Entrapment within a gel matrix;
(4) Containment behind a semipermeable membrane (hollow fibres, microcapsules, etc.);
(5) Intermolecular crosslinking of enzyme molecules.

Immobilization can be done with "live' or "dead" cells. In dead cells most of the life processes have been destroyed by more or less selective destruction while the enzyme(s) of interest is allowed to survive.

9. Materials used as supports (carriers) for immobilization have included both organic and inorganic materials (6a,6b). Cellulose, dextran (Sephadex), agarose (Sepharose), nylon, acrylamide-based polymers, and styrene-based polymers are typical examples of organic supports. Inorganic supports studied include porous and solid glass, diatomaceous earth, silica aluminas, nickel screening, stainless steel balls, sand, and titania. They often have better flow properties, and some are very inexpensive. Generally, the specific type of immobilization procedure and the support used are determined by the application of a given enzyme system.

10. A combination of unique catalytic properties of enzymes with their insolubility in an immobilized form has been used for a development of novel technological processes. At present these processes are related mainly to food and pharmaceuticals. This restriction of the areas of technological applications of immobilized enzymes could be explained in part by the existence of respective industrial chemical and microbiological processes with thoroughly outworked technology. Besides, in many cases enzymes continue to be industrially produced in relatively small quantities and are still rather expensive for the industrial applications, since the cost for immobilization and for respective carriers and chemicals is often too high.

11. It seems that in the near future only such processes using immobilized enzymes will be linked to industry, especially if the product can rarely be obtained without using enzymes, or if the cost of the product is so high compared with the cost of the initial materials (chemicals) that the difference can cover the expenses for their immobilization. That is why an interest is growing for the immobilization of enzymes by a simple adsorption on carriers and for using immobilized cells without an isolation of intracellular enzymes.

12. Up to the present time, only eight processes using immobilized enzymes or microbial cells have found industrial application:

(i) Production of high-fructose syrups by immobilized glucose isomerase.
(ii) Optical resolution of amino acids by immobilized aminoacylase.
(iii) Production of optically active D-amino acids by immobilized hydantoinase.
(iv) Production of L-aspartic acid by immobilized aspartase.
(v) Production of L-malic acid by immobilized fumarase.
(vi) Deacylation of penicillin G and cephalosporin derivatives by immobilized penicillin amidase.
(vii) Hydrolysis of lactose by immobilized lactase in dairy processing.
(viii) Production of glucose-fructose syrups from saccharose by immobilized invertase.

13. Besides these, there is a well-known process, already worked out on a pilot plant scale, which is apparently not far from its larger scale applications:

(ix) Production of glucose from starch hydrolysates by immobilized glucoamylase.

1. PRODUCTION OF HIGH-FRUCTOSE SYRUPS BY IMMOBILIZED GLUCOSE ISOMERASE

14. During the past 15 years, the major industrial application of immobilized enzyme technology has been the production of high fructose corn syrup (HFCS) by the isomerization of glucose to fructose, which is catalyzed by glucose isomerase of microbial origin. The isomerization process converts about 45% of the glucose (dextrose) in the corn starch hydrolysate, prepared in turn by enzymatic hydrolysis of wet-milled corn starch, into fructose.

15. The first successful commercial production of HFCS from starch hydrolysate using immobilized glucose isomerase was achieved in 1973 by the U.S. company Clinton Corn Processing, a division of Standard Brands, now Nabisco Brands. The process has created the largest new market developed in the past decade, including $ 40 million/year for immobilized glucose isomerase (for the further economic estimations see paragraphs 53-56).

16. The use of glucose isomerase began under a unique set of conditions: in 1974, the price of sugar in the U.S.A. increased sharply, making the cost of this conversion process competitive. By the time the cost of sugar had dropped again, the enzyme producers were able to reduce costs enough to remain competitive, and today the immobilized glucose isomerase is used to produce more than 3 million tons of HFCS yearly (4).

A. A Background

17. Today's typical process for making fructose syrups uses alpha-amylase enzyme to liquefy starch followed by glucoamylase enzyme to saccharify the hydrolyzed starch to the required 94 % dextrose content for isomerization to a mixture of glucose and fructose. The process is as follows (7). Typically a starch slurry of about 33 % dry solids is liquefied with a bacterial alpha-amylase at a temperature ranging anywhere from 80-110°C. This process usually takes 2-4 hr. Since the A'or alpha-amylase usually has a calcium requirement, the pH of the starch slurry is adjusted to 6-7 with calcium hydroxide. Acid hydrolysis is sometimes used, but the glucose concentration produced after saccharification is higher if enzymic hydrolysis is utilized. The hot slurry is then cooled to about 60°C at which point the liquefied starch is saccharified by treatment with a fungal glucoamylase which produces glucose from the liquefied starch. This process is generally carried out at a pH of 4-4.5 with a holding time of 24-90 hr depending on holding conditions and the amount of enzyme used. This generally converts the starch to 96-97% glucose on a dry basis. This crude syrup, high in glucose, is then decolourized with carbon and deionized with both acidic and basic resins. It is important to remove the calcium ions because they will inactivate glucose isomerase.

18. The crude glucose solution may then be concentrated or blended to bring its solids content to 40-50 %. Magnesium ions are generally added as they are required for almost all of the glucose isomerases used today. The pH of this crude glucose solution is adjusted to slightly alkaline (up to pH 8.5); the exact pH depends on the enzyme and the type of reactor. The glucose content of the starting syrup for the isomerase treatment is generally a minimum of 94% on a dry basis. This generally produces a fructose syrup containing a minimum of 42 % fructose (usually 43 %) and about 51 % glucose with no more than 6 % disaccharides or higher polymers (7). The syrup is then treated with activated carbon and deionized with a strong acid-cation exchange resin in the hydrogen form and a weak base-anion exchange resin in the free base form. This process should be carried out as rapidly as possible since the pH values reached with the ion exchange resins can range from 1.5 to 8, and this can induce chemical decomposition of the syrup components. The pH of the final product is generally adjusted to 4-4.5 which produces maximal stability. Finally the syrup is concentrated to 71% solids by evaporization during which process the pH is carefully controlled (7).

19. The sweetness of the resulting high fructose is comparable to that of common sugar (sucrose) or the invert sugar which is produced by acid or enzymatic hydrolysis of sucrose.

Sweetness of syrups (comparison for 15 per cent solution at 15°C)

Sugar (sucrose)	100
Glucose	75
Fructose	165
High fructose syrup (42 %)	100
Maltose	30-50
Lactose	20

Source: refs. 8,9

20. The resultant HFCS is the functional equivalent of sucrose in many applications. It has wide application as a nutritive sweetener in the food industry, especially in drinks and other beverages, desserts, baked goods, canning, and many other packaged products. For certain uses (e.g. cola beverages), HFCS with 55 % or 90 % fructose is preferred. This is made from the 42%-fructose HFCS by a separation process analogous to liquid chromatography (10).

21. The rapid growth in fructose demand in the last decade may be attributed to the following reasons:

- it can be used as an alternative to sugar because both are equally sweet.
- it can be produced at lower cost than sugar,
- it is produced from starch which is a common and widely accepted food mater
- it has a more refreshing taste than sugar,
- consumption of fructose reduces the risk of contracting diabetes and other metabolic disorders.

22. The amount of fructose which is attained by the isomerase reaction is determined by the equilibrium value of the glucose to fructose isomerization which gives roughly equal amounts of the two sugars. In order to obtain higher fructose content in the syrups, large - scale columns of strong acid-ion exchange in the form of calcium salt are used often as the stationary phase. Oligosaccharides come off the columns first, due to a molecular exclusion effect. These are then followed by glucose and finally fructose. Another possibility is to concentrate the syrup to a high enough solids content to allow crystallization of glucose from the concentrate. The remaining liquid is then enriched in fructose. It has been possible to make syrups up to 70 % fructose (dry basis) in this manner. The isolated glucose is recycled to the isomerization reaction. Finally, another approach is to complex fructose with borate compounds. This removes fructose from solutions and shifts the apparent equilibrium of the reaction. However, the cost of removal and recovery of the borate has prevented commercial adaptation of this process so far (7).

B. Commercial preparations of immobilized glucose isomerase

23. Clinton Corn Processing Co., developed the first industrial process of making HFCS, and used the microorganisms Streptomyces sp. to produce glucose isomerase. Novo Laboratories Inc., in contrast, selected the thermophilic microorganisms **Bacillus coagulans**, which, as the company indicated (10), produced glucose isomerase without requiring the addition of an expensive inducer such as xylose in the substrate, and could be grown in continuous culture without contamination and with high enzyme yield. In spite of the large number of organisms which could produce glucose isomerase (11), only a few high-producing organism strains are actually being utilized on a commercial basis.

Microbial sources of glucose isomerase presently being used for the commercial preparation of the immobilized enzymes, and the companies producing enzymes

Firm	Enzyme source
Clinton Corn Processing Co.	Streptomyces ribigenosus
	Streptomyces wedmorensis
Novo Industry	Bacillus coagulans
Gist Brocades	Actinoplanes missouriensis
ICI Americas, Inc.	Arthrobacter
Miles Laboratories, Inc.	Streptomyces olivaceus
Mi-Car Int.	Streptomyces olivaceus
CPC Int. Inc.	Streptomyces olivochromogenes
Corning Glass Works	Streptomyces olivochromogenes
Nagase	Streptomyces phaeochromogenes
Miles Kali Chemie	Streptomyces
Sanmatsu	Streptomyces
Snam Progetti	Streptomyces

Source: adapted from ref. 11

24. Most commercial organisms produce glucose isomerase intracellularly. The enzyme is generally produced by scaling up a submerged aerated fermentation in several stages. The three development stages of such a production are described in U.S. Patent 3,666,628: (a) slant development, (b) culture development - two substages, (c) final fermentation stage. Glucose isomerase can be recovered in two ways: (i) recovery of the microorganisms with the enzyme entrapped in the cellular mass, and (ii) recovery of the enzyme in the soluble form after lysin the cells (11). To prevent the loss of the enzyme from cells in the first case (as the result of the autolysis of the cells during isomerization) and to allow extended use of cells in fixed-bed reactors, the enzyme is bound to the cellular matrix by heat fixation or chemical fixation (11).

25. Commercial immobilized glucose isomerase preparations are generally produced by various companies in a granular form and a fibrous or amorphous form. Clinton Corn Processing Company, for example, produces both a fibrous form and a granular form of glucose isomerase. Each form is designed for a particular reactor technology. The fibrous form of Clinton's enzyme preparation has a large surface area with very high enzyme potency and is designed for use in shallow-bed reactors. The granular enzyme with lower potency is designed for deep-bed reactors. Novo Enzyme Corporation produces a granular isomerase suitable for deep-bed reactors, batch isomerization, and fluidized-bed operations. The immobilized enzyme preparation from Gist Brocades (Maxazyme^R) utilizes the organism **A.missouriensis** entrapped in cross-linked gelatin. This produces a softer particulate enzyme which can also be used in deep-bed reactors. ICI produces an immobilized enzyme preparation whereby Arthrobacter cells are flocculated by polyionic reagents. This also produced a rather soft granular particle that can be used in deep-bed reactors. Sanmatsu (Japan) produces a glucose isomerase by adsorption of enzyme on anion-exchange resin. This gives a high-potency, particular enzyme suitable for deep-bed reactors. Denki-Kagaku - Nagase Sangyo (Japan) produced an entrapped in high polymer isomerase (Sweetase^R) as hard granules for use in deep-bed reactors. The catalyst particles swelled to about double their original sizes (0.4-0.8 mm) during the isomerization reaction (8). Miles and Miles-Kali Chemie have produced a glutaraldehyde cross-linked preparation and a heat-fixed cellular preparation, respectively. Snam Progetti (Italy) produces glucose isomerase, extracted and partially purified from **Streptomyces sp.**, and entrapped in cellulose triacetate fibres. This is carried out according to the Snamprogetti's modification of the well-known technology of fibre wet spinning: an organic solution of fibre-forming polymer is emulsified with an aqueous solution of the enzyme, and the resulting emulsion is then extruded, through the holes of a spinneret, into a coagulation bath (12).

Commercially available immobilized glucose isomerase preparations

Company and patents	Immobilization procedure
Mi-Car Int. U.S. 3,625,828 3,654,081 3,779,869	Granular particles containing whole cells, cross-linked with glutaraldehyde
Clinton Corn Processing U.S. 3,623,953 3,788,945 4,376,824	Enzymes adsorbed on DEAE-cellulose via ion-exchange
Corning Glass Works U.S. 3,847,740 3,850,751 3,868,304 3,982,997 3,992,329	Enzymes adsorbed on controlled pore alumina carrier (0.46 mm D; APD 140 - 220 A); carrier can be regenerated
Gist Brocades U.S. 3,834,848 3,838,007	Whole cells entrapped in gelatine followed by cross-linking with glutaraldehyde
ICI Americas, Inc. U.S. 3,645,848 3,821,086 3,935,068	Flocculating agent used to immobilize enzymes within cells; paste then extruded and dried into cylindrical pellets
NOVO Industri BR 1,362,365 1,381,387 U.S. 4,025,389	(a) Enzymes mixed with inorganic diluent and formed into solid spheres (b) Lysed cells cross-linked with glutaraldehyde
Miles Laboratories, Inc.	Glutaraldehyde cross-linked whole cells
CPC International, Inc. U.S. 4,343,902 Canada 998,344	Adsorption on alumina (porous) or other ceramic carriers or ion-exchange resin
Miles-Kali Chemie	Heat-fixed cells cross-linked with glutaraldehyde
Sanmatsu	Adsorption on anion exchange resin
Snamprogetti U.S. 3,964,970	Enzyme entrapped in cellulose triacetate fibres by means of fibre wet spinning
Mitsubishi Chemical Industries	Adsorption on undisclosed synthetic anion exchange resin

Agency of Industrial Science and Technology (Japan)	Heat-fixed cells
Denki Kagaku Kogyo	Enzyme adsorbed on cation exchange resin having quaternary pyridine ring
P.J. Reynolds Tobacco Co.	Cells coagulated with flocculant
Kyowa Hakko Kogyo	Enzyme adsorbed on phenol formaldehyde resin Duolite A7
Denki Kagaku-Nagase	Whole cells entrappment into a high polymer (Sweetase^{R}),and granulated up to 0.4-0.8 mm particle sizes

Source: adapted from ref. 11-14.

26. The first main conclusion following from the above table is that among commercial preparations of immobilized glucose isomerase there is actually no covalently bonded enzymes. Glucose isomerase is mainly either in adsorbed form by means of ion exchange resins or porous inorganic carriers (alumina, ceramics) or inside of whole cells entrapped in a polymer matrix usually cross-linked with glutaraldehyde or other bifunctional reagents. Even Corning Glass Works which has developed well-known procedures for covalent immobilization of enzymes on inorganic carriers, in this particular case uses immobilization by adsorption. The main reason for this is that immobilized glucose isomerases which have been produced by adsorption of soluble enzyme onto a solid carrier can generally be regenerated and reloaded with fresh enzymes after much of the initial activity has been depleted. It is usually economically advantageous to do so especially when using an expensive carrier.

27. The second main conclusion is that in many cases whole cells (heat-fixed or entrapped into polymer matrix) have been used instead of the enzyme isolated. This could be explanted both by lesser stability of isolated (extracellular) glucose isomerase (14) and by higher cost of isolation and immobilization of enzymes in comparison with the use of whole microbial cells.

28. These two points (par. 26 and 27) are illustrated below in the form of a comparison of the general procedures of the enzyme immobilization with respect to several important commercial properties (11).

Expected properties of immobilized glucose isomerase prepared by various procedures

Immobilization procedure	Expressed activity (observed/ bound)	Potency (activity/ weight)	Carrier cost	Reusability of carrier
Immobilized in bacterial cells	High	Average	Low	No
Adsorption on insoluble carrier	High to low	High	High	Yes
Entrapped in insoluble matrix	Low	Average to Low	High	No
Covalently bound to insoluble carrier	Low	Low	High	No

Source: ref. 11

29. The increase in HFCS penetration of the world sweetener market has been made possible in part by the evolution of immobilized glucose isomerase technology and improvements in enzyme characteristics. This dynamics can be traced with the example of Novo Laboratories Inc. which subdivided various types of immobilized glucose isomerase preparations developed by the company into the preparations of three generations (10). These do not include their first of a series of commercial glucose isomerases marketed under the brand name Sweetzyme^{R} which was a spray-dried soluble product introduced in 1973. It had a high production cost and was used commercially for only a short time. When Novo's first immobilized form of the enzyme was introduced in 1974, its advantages became readily apparent and use of the spray-dried soluble form was subsequently discontinued (10).

30. The steps in the production of immobilized glucose isomerase of "three generations" (and for three types of reactors) are shown in Figure 1 and described below (10). For production of the first generation enzyme (1974), in the form of powder, the cross-linking agent selected was glutaraldehyde, which immobilized the cell protein. The initial product of the cross-linking was a semi-gelatinous mass of pudding consistency. After drying, grinding and screening, the final product has a particle size range of 100-350 microns. The product was designed for use (and reuse) in a batch reactor (Fig.2).

31. The first-generation immobilized enzyme had several limitations. The particles gradually decreased in size as a result of the shearing effect of the agitator blades. This led to an overall decrease in settling rate and thereby to a slowing down of the process. According to Novo's data, decrease of the size particles of the immobilized enzyme from 0.35 to 0.25 mm and further to 0.10 mm leads to a decrease of the terminal velocities of settling particles from 9.5 to 5.0 m/hr and further to 0.8 m/hr. In addition to the degradation of the particle, there occurred a thermal denaturation of the enzyme, leading to a decrease in available activity. This could be compensated for, either by prolonging the reaction time or by adding new enzyme to the isomerization reaction tank prior to the next cycle. Normal make-up was about 5 % per reuse. Finally, the long holding time required for the isomerization with the first generation enzyme required in turn, operation at a relatively low pH to avoid excessive colour and by-product formation. At this pH, the addition of cobalt salts was necessary to activate the immobilized enzyme. These drawbacks of the powder form of the enzyme and the batch reactor led to the development of immobilized enzyme of the second generation, suitable for continuous column reactors, where the reaction could be run at a neutral to slightly alkaline pH, since the syrup contact time was drastically reduced, and no cobalt addition was required.

32. The second-generation immobilized enzyme for column use has been prepared by the extrusion of the wet cross-linked enzyme mass through a small orifice (< 1 mm diameter) and cut into short cylindrical particles, which were then dried and screened, as shown in Figure 1. This product did not show suitable pressure-drop characteristics, because upon continued operation the particles had deformed and the pressure drop had risen dramatically. This was overcome by modifying the immobilized enzyme preparation by incorporating powdered alumina to increase the density of the particles and allow higher flow rates in the upflow mode (10). Industrial performance was entirely satisfactory, however, and the deviation from the laboratory data was ascribed to variations in enzyme particle size and to density and flow-distribution problems.

33. For the preparation of the third-generation immobilized glucose isomerase, which should have been able to resist deformation at constant syrup flow for fixed-bed operation, an important change in the immobilization process was made. The cell slurry was homogenized prior to cross-linking as shown in Figure 1. The homogenization disrupted the cell membranes and exposed more protein surface for reaction with the cross-linking agent. The result was an enzyme with stronger particles that resisted deformation. The improved type of the enzyme preparation also contained additives such as magnesium oxide and dextrose, which were incorporated after cross-linking but before extrusion. The magnesium oxide helped to minimize pH drop during isomerization, and the dextrose dissolved during operations, resulting in a more porous matrix and lowering diffusion resistance (10). The enzyme has been shipped to the user in a dry granular form which must be hydrated in syrup prior to use. During the hydration, the enzyme particles expand in size by a factor of about two. In the operating conditions (pH 7.5-8.0, temperature 55-60°) half-lives of the enzyme are usually more than 75 days.

C. Technological characteristics of the processes

34. The literature contains few data on the commercially available glucose isomerase technologies. Listed below are some data on the reactor configuration normally used, and where available, basic performance data. Although each system listed employs a different enzyme source (see above), a different level of enzyme purity, and a different immobilization technique, there appears to be relatively little difference in performance of the system for which data are available (15).

35. Application of deep-bed reactors is described by major commercial manufacturers of immobilized glucose isomerase. A deep-bed reactor is usually simpler in design than a shallow-bed reactor and therefore may require a lower capital expenditure. Flow may be upflow or downflow through the reactor. Column reactors operating in the down mode appear to be most popular. Heights up to five meters appear to be feasible. Upflow in a column had been reported to give lower productivity, probably due to wider distribution of the syrup residence time. Precautions must be taken with upflow to prevent loss of immobilized enzyme through the top of the reactor, and flow control is considered to be more critical than for downflow (11). The recommended temperature and pH are almost identical (60-65°C and 7.0-8.5 respectively). The initial substrate is 40-50 % dry solids (92-94% dextrose, dry basis, 6-8 % dry basis polysaccharides).

Technological characteristics on the immobilized glucose isomerase systems

Company	Reactor configuration and basis performance data
Mi-Car Int.	Column reactor
Clinton Corn Processing	Shallow bed reactors; the immobilized enzyme constitutes a thin bed of 2.5-12.5 cm depth. Several of these beds are staged to form a multibed processing unit. The multiple filter leaves in either a vertical or horizontal tank, are packed with beds at a bed-depth: bed-width ratio of about 0.02-0.05.
Corning Glass Works	Column reactor. The half life time of the enzyme is 40 days.
Gist Brocades	Stainless still column reactor to 6 m bed depth, 1.5 m diameter. The half-life time of the enzyme 21 days.
Sanmatsu	Column reactor. The half-life time 30-50 days. Glucose concentration 45-50 %, temperature 55-70°C, pH 6.5-8.5.

ICI	Columns to 5 m bed depth.
NOVO	Columns to 4.5 m bed depth. Conditions: 60°C, pH 7.5-8.0. The half life time 75 days.
Snam Progetti	Tubular and radial reactors. Fibres contained glucose isomerase (entrapped) are placed in an ordered way parallel to the long axis of the column (tubular reactor, 0.2-0.25 kg of dry fibres per litre of reactor volume) or are rolled up around a perforated pipe in an ordered way, as on a bobbin (radial reactor, 0.35 kg of dry fibres/l).The half-life time 70 days.
Denki Kagaku Kogyo	A battery columns reactor. Raw material (dextrose, 50 wt % solids) -pH 4.0-4.5. Product (at the reaction column outlet) - pH 7.5.

Source: adapted from ref. 8,12,15.

36. In continuous production of isomerized sugar by the immobilized enzyme process, raw material purity influences catalyst activity and therefore productivity. Thus, raw material (dextrose syrups) should be refined as much as possible. Impurities contained include calcium ions, peptides, oxygen, oxidation products, etc. According to the data by Chiba Plant, Denki Kagaku Kogyo K.K. (8), if a high purity substrate such as crystalline glucose is used as the initial substrate, the productivity of the reactor (the yield of solid high fructose syrup per unit weight of catalyst produced to the point at which the catalyst activity has decreased to a quarter of the original level) reaches 4000 kg/kg of immobilized enzyme in 100 days, and the half-life time for the catalyst is equal to 50 days. If purified dextrose of lower grade is used, however, the half-life time decreases to 20 days, and the productivity of the reactor decreases to 1,500 kg fructose/kg of immobilized enzyme. In general, productivity for commercially-used immobilized glucose isomerases, described by various enzyme manufacturers and used under their recommended operating conditions, varies from 1 to 9 tons of HFCS (dry basis) per 1 kg of the immobilized enzyme.

Productivity of some commercial immobilized glucose isomerases

Manufacturer	Productivity (tons 42 % HFCS/kg enzyme)
Clinton Corn Processing Co.	7.2-9.0
Gist Brocades N.V.	1.78
ICI Americas, Inc.	2.0
Miles Kali-Chemie	1.0
Novo Industri A/S	1.0-1.6
Novo Laboratories, Inc.	4.0
Snamprogetti	5 - 6
Mi-Car Int.	2.0

Source: refs. 10,11,15

37. A large number of reactor designs has been described for application with immobilized glucose isomerase (six different designs, according to one particular classification, i.e. batch, packed-bed, continuous-flow stirred-tank, continuous-flow stirred-tank/-ultrafiltration membrane, and other, including recycle reactors and tubular reactors with enzymatically active walls) (11). However, most glucose isomerase reactors now in commercial operation are of the packed-bed type. Novo Industri has described batch reuse of their Sweetzyme Type A, a glutaraldehyde cross-linked homogenate of **B.coagulans**. Although they subsequently found performance advantages with a continuous system, they reported in 1976 that Sweetzyme Type A had been used commercially in large-scale batch reuse since 1974 (cit. in 11). Gist-Brocades has also described conditions for reuse of their Maxazyme GI-Immob. in a batch reactor.

38. Numerous investigators have compared performance and economics of a batch versus a continuous reactor system. It was found that usually in a packed-bed reactor the concentration of active glucose isomerase is high compared to a batch reactor and contact time between substrate and enzyme is relatively short, usually 2-4 hr in comparison with 20-60 hr for a batch reactor (11). The short contact time helps to minimize formation of coloured materials and nonfructose isomerization compounds. Then, enzyme usage (comparative amounts of enzyme activity) has been found to be considerably higher (1.4-4.0 times) in all batch reactor cases than for a continuous packed-bed reactor, primarily due to loss of active enzyme through multiple batch recovery operations (11).

39. U.S. Patents 3,847,740 and 3,847,741 disclose a process for regulating production by means of temperature control as well as for increasing the productivity of the enzyme. One example demonstrates that by increasing the temperature in the reactor in 2°C increments from 60° to 70°C over a period of 14 days, the enzyme productivity is increased 42 % over a 14-day isothermal run at 60°C.

40. Novo Laboratories Inc. has been using the 'first-generation' immobilized glucose isomerase (see para. 30) in a batch reactor Fig. 2). The enzyme was typically added to a tank containing purified high-dextrose syrup (> 93 %, dry basis) adjusted to certain conditions (pH 7.0, temperature 60-65°C, Co^{++} $3.5x10^{-4}M$, $MgSO_4.7H_2O$ 0.1-1.0 g/liter). The typical reaction time was 20-24 hr, after which the enzyme was recovered by allowing the particles to settle and drawing off the supernatant liquor. The entire process was then restarted. The limitations of this mode of the process operation were described in paragraph 31.

41. Glucose isomerase of the second generation, that is crosslinked with glutaraldehyde and extruded (see para. 32) has led to non-satisfactory pressure-drop characteristics of the column reactor in the downflow operation mode. Eventually, the entire enzyme bed would compress to the point that flow was drastically reduced and the operation had to be stopped. Therefore, Novo Labs at that stage of the development (1975) attempted to use an expanded - or fluidized-bed reactor, in which the dextrose syrup flowed into the bottom of the column through a flow distributor and the immobilized enzyme was suspended in the flowing syrup (10). In the fluidized-bed operation, the substrate solution passed upward through the enzyme bed. In this operation mode the most critical factor was uniform flow distribution. This was achieved in commercial reactors by using a shallow bed of heavy inert material (alumina) on a screen.

42. With the third-generation immobilized glucose isomerase having stronger particles that resisted deformation (para.33), the enzyme slurry after the hydration transferred to the column reactor and ran in an upflow (fluid-bed) mode for about 12-24 hr. This permitted the enzyme to come into equilibrium with syrup at column operating condition without compacting. After this "fluid" period, the bed was settled briefly, then ran in the downflow mode. This mode of operation is currently in wide industrial use in many parts of the world (10). The enzyme bed height at the optimum operating conditions is of 5 m maximum, diameter 1.5 m maximum (height:diameter ratio is of 3:1 minimum), temperature 55-60°C at inlet, pH 7.5-8.0 at 25°C, dextrose concentration at inlet 94 % minimum (dry basis), feed solids 45 %, Mg^{++} concentration equals $20xCa^{++}$ concentration. With these operating conditions, half-lives are usually more then 75 days (10).

43. According to Novo's recommendations (10), to achieve high enzyme productivity (in their particular case, this is above 4,000 kg of dry solids/kg of enzyme, at 45 % conversion of dextrose to fructose. See also paragraph 36) on a continuing basis, careful attention must be paid to details of plant design and operation. For example, in reactor design, flow distribution is important for operation over a wide range in flow rate. The enzyme is often used until the flow rate (activity) drops to 10 % of the initial flow. Feed purity is of great importance to avoid cumulative enzyme poisoning (cf. paragraph 36). Filtration, decolourization (active carbon treatment), deionization, and evaporation must be designed and operated carefully. It is important to provide a purified feed of constant composition and consistent parameter control.

44. There are data available on the isomerization process by Kyowa Hakko Kogyo Co., Ltd (16). Glucose isomerase used in the process was immobilized on a phenolformaldehyde resin Duolite A7. The flow chart of the process is shown in Figure 3. 40 % (w/v) glucose solution kept at 60°C was continuously fed through the immobilized enzyme columns in series at 60°C and pH 8.2. Before feeding, the substrate solution was passed through pretreatment columns which contained Duolite A7 (buffered) to eliminate some impurities and air-bubbles. The effluent solution from the immobilized enzyme column was then passed successively through a cation exchanger (Diaion SK-1A, H-type) and an anion exchanger (Diaion WA 20, OH-type) in order to eliminate salts. Microbial contamination was not observed throughout the whole operation period probably because of the high glucose concentration (40 %) and the high operational temperature (60°C). The half-life of the enzyme catalyst at the operating conditions was 34-40 days, depending on the flow rates. On the company's data, 1 litre of the immobilized (an adsorption type) enzyme can isomerize 288 kg of glucose within 30 days at these operating conditions shown above. On the other hand, 1 litre of a covalently immobilized enzyme (using triazinyl chloride as a coupling agent) can isomerize 576 kg of glucose during the same period.

45. In Clinton Corn's approach, the flowsheet of which is shown in Figure 4, the immobilized enzyme constitutes a thin bed of 2.5-12.5 cm depth (17). Several of these beds are staged to form a multibed processing unit resembling a pressure leaf filter (see also paragraph 35). A major advantage of having more than one bed is to minimize the effect of channelling that easily occurs in the shallow beds. For the bed dimensions Clinton Corn recommended a depth-to-width ratio of about 0.02 to 0.05 (11,17).Single beads can be removed during processing without interruption of the process flow for regeneration. Carbon treatment and ion exchange purify the 42 %-fructose effluent, and evaporation concentrates it to syrup. Enzyme half-life, according to Clinton Corn, is equal to "several hundred hours" (17).

46. In the mid 1970s the world's first manufacturer of isomerization sugar (by means of soluble glucose isomerase) was also the biggest operator in Japan, Sanmatsu Kogyo in co-operation with Mitsubishi Chemical Industries Ltd, and Seikagaku Kogyo Sanmatsu Kogyo developed an ion exchange resin which selectively adsorbs glucose isomerase "without affecting its activity" (14), and signed a license contract in which a commercial plant was put to test operation in October 1975. Having confirmed good test results, Sanmatsu Kogyo decided to switch its whole production equipment in early 1976, and later during that year, Sanmatsu factories in Chiba and Fukuoka were remodelled to use Mitsubishi's technology. Since then commercial operation of these factories has been introduced. The production process may be summarized as follows (14). Amicrobial Microbial producer of glucose isomerase is filtered for culture liquor separation, repulped in water and treated with some additives for several hours to extract the enzyme. After separation of the residual cells, enzyme liquor is placed in contact with ion exchange resin, which adsorbs almost all of glucose isomerase in a matter of several hours. Since the carrier selectively adsorbs glucose isomerase, no refining is necessary for the enzyme liquor. The isomerization itself takes place in a simple column packed with the immobilized enzyme, at a glucose concentration of 45-50%,reaction temperature 55-70^{o}C, pH 6.5-8.5, space velocity (initial) 1.5-4 hr^{-1}. The glucose conversion level is 45%. At these operating conditions the half life of the immobilized enzyme is 50 days if crystalline glucose is used as raw material, or less, down to 30 days, if raw material is saccharified glucose solution subjected to conventional purification. Isomerized glucose syrup is almost colourless with few by-products. Simple treatment of the syrup with ion exchange resin completely desalts and decolourizes the product. Immobilized enzyme after being used is washed away from its carrier in the reactor through a regeneration step employing 'some chemical solutions'. The regenerated carrier is reused for isomerization after adsorbing new active enzyme.

47. According to (18), Chinese biotechnologists used glucose isomerase from Streptomyces roseoruber, adsorbed on a strong basic anion exchange resin 290 (made by Nankai University, China), for making HFCS. Pilot plant scale experiments have been carried out with 1.25-2.2 tons of HFCS produced per kg of dry immobilized enzyme.

48. In the Snam Progetti (Italy) process glucose isomerase entrapped in fibres was used for the isomerization of industrial glucose solutions. Under the operating conditions (65°C, 50 % w/w glucose syrup) the half-life of the immobilized enzyme preparations was about 70 days. The product was colourless, and no activated carbon treatment was required. The best process performances have been achieved with tubular and radial reactors (12). In the tubular reactor the packing was obtained with the fibres placed in an ordered way parallel to the long axis of the column, a packing degree in the range of 0.2-0.25 kg of dry fibres per litre of reactor volume resulted in a stable catalyst bed practically uncompressible and with negligible channelling and relatively low pressure drop. On the other hand, the radial reactor fits very well with the filamentous structure of the fibres. It was prepared by rolling up the fibres around a perforated pipe in an ordered way, as on a bobbin; the reactor mixture flows from the holes of the central pipe, passes through the fibre layer where the enzymic reaction takes place, and exits. Packing degrees up to 0.35 kg of dry fibres per litre of reactor volume were reached, making it possible to increase the efficiency of the fibre-entrapped glucose isomerase and to decrease the residence time. According to Sham Progetti, the radial reactors have the great advantage of being easily prepared by using standard equipment of the textile industry, such as winding machines. The total productivity during two half-lives of the immobilized enzyme in the reactors was around 5-6 tons of HFCS (dry mass) per kilogram of fibres (12).

49. Figure 5 shows a flowsheet for the Hungarian industrial process with a processing capacity of 400 tons of native corn daily, or 120,000 tons/year (19). The plant based on the wet milling of maize was built in 1983 in Szabadegyhaza in Hungary, and has been operating since then. The first step of process is the continuous enzymatic liquefaction of the starch slurry, based on the technology of Miles/DDS-Kroer and using thermostable alpha-amylase Optitherm^R of Miles Kali-Chemie. The hydrolysate leaves the dextrinizing tank with a DE value of 15. The liquefaction is followed by the enzymatic saccharification at 60°C and pH 4.5 in batch mixing tanks of 200 m^3. For this step, the Aspergillus niger glucoamylase of Miles Kali Chemie (Opti- dex^R) is used. After a saccharification time of 60 hr, the DE value of the resulting glucose solution is 96-98. The glucose solution is purified by separation from the germ oil, by rotary drum filtration in the presence of active carbon, and by full ionization with ion exchange resins. Following the evaporation step, pH and temperature are corrected, magnesium "ions are added, and the solution is pumped for continuous isomerization into columns packed with immobilized glucose isomerase Takasweet^R, Miles-USA.

50. In the Hungarian process two columns are fed by down-flow and operated in parallel; new columns are put into operation at the necessary time when the activity of the immobilized glucose isomerase decreases below the control level. The reaction process proceeds at 60-62°C, pH 7.8, with the productivity of 2.5 tons of the syrup (dry mass) per kg of the immobilized enzyme; the enzyme half-life is 40 days. The bed volume of enzyme catalyst in the column is 20 m^3, the overall consumption of the immobilized enzyme equals 35 tons/year (20). The isomerization is followed by ion exchange, filtration with active carbon and finally by concentration in a 4-stage falling film evaporator to 71 % DS with a standard composition of 42 % fructose, 52 % glucose and 6 % malto-oligosaccharides.

51. Novo's plant-scale design recommendations (21), based on the criteria for their Sweetzyme^R such as initial enzyme activity 200 IU/gm, half-life 825 hr, running time 2 half-lives, and wet bulk density 0.3 gm/cm^3, are as follows:

Plant capacity	400 tons dry solids/day
Syrup concentration	40 % w/w
Syrup flow	35.6 m^3/hr (average)
Bed volume	50 m^3
Number of reactors	6
Reactor internal diameter	1.45 m
Reactor bed height	5 m
Average linear flow velocity	3.6 m/hr
Initial linear flow velocity	6.5 m/hr
Enzyme consumption	220 kg/day
Operating cycle	275 hr
Flow variation	11 % of average

As Novo indicates, similar data have been applied as the basis for the design and engineering of a number of fructose syrup plants in Europe, the Far East, the United States, Canada, and Latin America (21).

52. It is worth mentioning that in 1977-82 Cetus Corporation, the U.S. R&D company headquartered in Berkeley, California, developed a patented two-step process for making 100 % pure fructose from glucose syrups (U.S. Patents 4,247,641; 4,284,723), which was "ready for scale-up" (22). The project had been funded by some $ 8 million from the Standard Oil Company of California (Socal), which, having retained its 17 % corporate investment in Cetus, had left the latter with full title to all patents and know-how generated. Cetus' proprietary new technology converts the glucose to 100 % fructose in two steps: one enzymatic (the oxidation of glucose to D-gluconolactone by means of immobilized glucose oxidase derived from **Polyporus obtusus**), the other chemical - the reduction of the gluconolactone to fructose by hydrogen using palladium as catalyst. The hydrogen peroxide appearing as by-product of the enzymatic glucose oxidation is utilized to transform ethylene or propylene into the corresponding oxide by the consecutive action of the two other enzymes, haloperoxidase and halohydrin epoxidase (23,24). The process worked out for the transformation of glucose was termed by a Cetus' representative as "the third-generation technology for fructose production" (22). Cetus expected to develop the process to a full-scale facility of 250,000 to 500,000 tons annual capacity. Thus, so far, unsuccessful commercialization of the process may indicate insurmountable obstacles were encountered in translating the coupled reaction to an industrial process (24).

D. Economic estimations

53. The increase in high fructose syrups penetration of the world sweetener market has been made possible in part by the evolution of immobilized glucose isomerase technology and improvement in enzyme characteristics and process conditions. Each improvement has shown better cost effectiveness. Shown below, for example, are the dynamics of decreasing the relative enzyme cost for producing high fructose corn syrup by Novo (10).

Relative cost of immobilized enzyme for producing HFCS

Year first used	Type of reactor	Relative enzyme cost
1974	Batch	3-4
1975	Fluid-bed	2
1975	Fluid-bed	2
1976	Fixed-bed	1.1
1978	Fixed-bed	1.0

Source: ref. 10.

54. There are economic estimates available for the Kyowa Hakko process of isomerization of glucose by means of glucose isomerase immobilized on phenol-formaldehyde resin (see paragraph 44), in comparison with the data for the native (soluble) glucose isomerase (16). The cost estimate was based on a process in which 50 tons of glucose were converted monthly to isomerized sugar mixture containing 45 % fructose. In order to isomerize 1,000 kg of glucose, 21 kg of glucose isomerase containing fungal mycelia were required in the batch process using native enzyme. In the case of the immobilized enzyme systems 9.8 l of immobilized enzyme could be obtained from the same amount of mycelia. Being adsorbed on Duolite A7, 9.8 l of the immobilized enzyme could isomerize 2,822 kg of glucose within 30 days when it was operated at 60°C using 40 % glucose solution as substrate. It was presumed that the immobilized enzyme system was operated using two columns in series and that the older column was replaced with a fresh column each 30 days. Concentrations of $MgSO_4$ and $CaCl_2$ required in the immobilized glucose isomerase systems were 1/10 and 1/20, respectively, compared with the native enzyme systems. This decreased the volume of ion exchange resins required for deionization of the isomerized sugar mixture. Colouration of the isomerized sugar mixture in the immobilized enzyme system was much smaller, i.e. about 1/100, compared with that of the native enzyme system. This also decreased the cost for decolouration. Labour costs were much lower in the immobilized enzyme system compared with the batch system. All these factors were taken into consideration, and relative tentative costs of these two systems were calculated. The results are shown in Figure 6. Assuming the variable cost in the batch system as 100, the relative cost of Duolite A7 system was calculated as 61.5.

55. Another set of economic estimates was prepared by Purdue University (25). The object of their study was to find the optimum cycle time and relative costs of operation for soluble and immobilized glucose isomerase. The latter was covalently bonded "to an expensive but good carrier", agarose activated by cyanogen bromide. The method was to bind the enzyme to the carrier, measure activity and stability of the preparation, and design a plant to process 450 ton/year of dry HFCS. Optimum cycle time was calculated to give minimum annual cost

of the reaction. One-year carrier life was assumed. A batch process (soluble enzyme) was also designed for comparison. As shown below, for the batch process, the enzyme is the major cost. For the immobilized -enzyme process, the expensive carrier and reagent are the major cost. Even though these are high, the cost of the continuous process is less because the enzyme is expensive and it is used in the continuous process for longer periods than it can be used in the batch process. It is worth mentioning here that the actual isomerization step, including cost of the enzyme, usually accounts for less than a quarter of the total production cost; pretreatment of feedstock and product clean-up accounts for the remainder.

Economic estimations for producing 450 ton/year (dry mass) of high fructose syrups. Glucose isomerase was bound to agarose by cyanogen bromide. One-year carrier life was assumed.

	Batch, soluble enzyme	Continuous, immobilized enzyme
Reaction time (arbitrarily set)	20 hr	-
Optimum running time, days	-	29
Annual costs:		
Enzyme	$ 1,000	$ 37
Carrier	-	1.040
Reagent	-	1.580
Reactor shell	300	185
Reaction cost, cents per pound	1.3	0.28

Source: ref. 25

56. In Hungary, economic estimates made in the beginning of the 1980's have shown that, although the sugar yield of beets per unit of weight was much higher compared to that of maize, the total money value of products when using maize should be at least 25 % more. It was shown that the raw material cost of sugar from maize was 3.53 Hung. forint (1986 U.S. $ 0.28) per kg, whereas beet sugar cost 5.40 Hung. forint (1986 U.S.$ 0.44) per kg. As a result, a new maize processing plant was built in Szabadegyhaza, Hungary (see paragraph 49 and 62) with a processing capacity of 400 tons of native corn daily (120,00 ton/year). The investment costs for the plant were reported to be about 2.1 billion Hung.forint (1986 U.S. $ 169 mil.), and pay-out time, based on the standards of the national economy, was calculated as 7.3 years (19).

E. Scale of the processes

57. For industrial production of high fructose syrup, capacities ranging from 30 to 100 tons (8) or 400 tons (21) a day (dry solids) are considered best. Due to the rapid development of the new technology using immobilized glucose isomerase, high fructose syrup from corn starch is a rapidly expanding business, and undoubtedly represents

the most successful use of an immobilized enzyme in food chemistry. Currently, the United States accounts for most of the world's HFCS production utilizing immobilized glucose isomerase. Japan is the second-largest producer. The growing utilization of HFCS in these two countries is shown below.

Annual production of high fructose corn syrup in the USA and Japan

Year	U.S.A. (thousands of tons)	Japan (thousands of tons)
1972	-	-
1973	136	30
1974	363	-
1975	645	75
1976	908	130
1977	1135	216
1978	1585	-
1979	1680	-
1980	2300	300
1981	2680	-
1983	3250	-

Source: refs. 8,10,14,23,26.

58. It has been reported (8) that by the end of the 1970's high fructose syrup reached a level of 10% of the demand level of sugar consumption in Japan, which was estimated at being between 2.4 and 3 million tons a year. In the U.S., usage of HFCS in 1978 already has reached 6 kg dry basis per person (12% of sugar consumption) (11) and a steadily increasing per capita usage is projected to reach 30-40% in 2000. Significant commercial production facilities are also in operation in Canada, Argentina, Austria, South Korea, and several European countries (see below). According to (27), in 1982 HFCS accounted for 4 % of the world's production of caloric sweeteners. Penetration of world market of industrial sugars by HFCS had reached as much as 32 % in 1981, and 36 % in 1982 (10).

Per capita nutritive sweetener consumption trends in USA

Year	Total, kg	Sucrose, %	Corn syrups (other then HFCS), %	HFCS, %
1970	55.0	84.1	15.9	-
1972	56.3	82.8	16.3	0.9
1974	55.3	79.2	18.6	2.2
1976	56.6	75.9	18.7	5.5
1978	57.8	73.1	17.3	9.7
1980	58.2	68.0	15.6	16.4

Source: cit. in ref. 28

59. The glucose isomerase market is dominated by three manufacturers: Novo Industri of Denmark, Gist Brocades of the Netherlands, and Miles Laboratories of the U.S.A. A rough estimate of the 1983 market was 1,625 tons of glucose isomerase which in turn has created a $ 40 million/year market for the immobilized enzyme. In 1982 approximately 2.15 million tons of 42 % HFCS and 1.45 million tons of 55 % HFCS was produced (29). Average productivity for commercially used immobilized glucose isomerase is about 2,000 kg of HFCS per kg of the enzyme, and HFCS manufacturers produced an estimated 3.7 million tons of the product in 1980. It is worthwhile to mention also that the U.S. fructose market in 1982 amounted to about $ 11 billion a year and has been marked by high price stability (22).

60. Today, several companies are producing fructose corn syrup in the United States with an estimated production volume in 1983 of over 3 million metric tons. The producers and brand names of their products are listed below. The economics of fructose syrup production in the United States have been compared to those for beet sugar production with the conclusion that there is an advantage to processing corn starch into fructose syrup in the U.S.A. (11).

42 % fructose corn syrup producers (U.S.).

Company	Brand name
American Maize Products Company	TruSweet^{TM}
Amstar Corporation	Amerose
Archer Daniels Midland Corn Sweeteners	Corn Sweet^{TM}
Cargill, Inc.	ISOCLEAR
Clinton Corn Processing Company	ISOMEROSE^{R}
CPC International, Inc.	INVERTOSE^{TM}
The Hubinger Company	HI-SWEET^{R}
A.E.Staley Manufacturing Co.	ISOSWEET^{R}

Source: ref. 11

61. Fructose syrup is produced in Japan (paragraph 57) and Europe although the present markets are somewhat limited, compared to that of the United States. In Europe, where the product is known as isoglucose, and particularly in the European Economic Community, development of fructose syrup production has slowed down due to the strong political influence of the sugar industry and a subsidy on exported beet sugar (11). Another constraining factor is that European corn is difficult to process by wet milling, and therefore a major part of the corn needed for fructose syrup production would have to be imported without the luxury of a subsidy (11). Even so, 1976 production of fructose syrup in Europe was estimated to be about 100,000 metric tons (United Kingdom, 35,000 tons; Spain, 25,000 tons; West Germany, 21,000 tons; Belgium, 14,000 tons; the Netherlands, 10,000 tons), 1980 production - about 0.75-1.0 million tons. Since that time additional fructose syrup plants have been constructed in France (Societé des Products du Maise and Roquette Freres), Ireland, Italy (Liquichemica under license from Miles Laboratories and Cargill), the Netherlands, United Kingdom (in Tilbury, under a joint venture between Schotten/Honig of the Netherlands and Tate & Lyle of the U.K.), Yugoslavia (under a joint venture of AIPK Poljoprivreda, Miles Laboratories, and MI-Car International, a Miles affiliate), Canada (two plants, one under

joint venture of John Labatt Ltd., Toronto and Redpath Industries Ltd., a unit of Tate & Lyle, Ltd; and the other by Canada Starch Co., a unit of CPC International). Total world production of high-fructose syrup by 1980 has been estimated to be about 3.7 million metric tons (11).

62. In 1983 in Szabadegyhaza (Hungary), a new maize processing plant was put into operation with a processing capacity of 400 tons of native corn daily (120,000 ton/year). The yearly production is at present: 50,000 tons of HFCS (42% fructose), 30,000 tons of protein feed, and 20 million litres of ethanol. The plant's annual consumption is 95 m^3 of thermostable alpha-amylase, 90 m^3 of glucoamylase, and 35 tons of immobilized glucose isomerase (19, 20).

63. As a recent example in the area, in February 1985 Cargill Incorporated began full operations at its new $ 100 million wet-corn mill in Eddyville, Iowa. The plant, capable of producing up to half a million tons of HFCS, employs 100 people and processes more than 20 million bushels of corn (30).

II. OPTICAL RESOLUTION OF AMINO ACIDS BY IMMOBILIZED AMINOACYLASE

64. Utilization of L-amino acids for medicine, food, and animal feed has been developing rapidly in recent years. In 1982, the world production of amino acids was estimated to be 500,000 tons, with a market value of about US $ 1.3 billion (31). From this amount 300,000 tons were used in food industry as mono sodium glutamate; 100,000 tons of DL-methionine were used as animal feed additives, and 40,000 tons of L-lysine as food and fodder constituent.

65. These data are generally consistent with those for 1979, according to which the world production of amino acids amounted to 424,340 tons, of which 270,000 tons were L-glutamatic acid, 100,000 tons were DL-methionine, and 29,000 tons were L-lysine (32). The biggest producer of methionine is Degussa AG, the Frankfurt (West Germany) chemical and precious metals company, which produced 75,000 tons/year of that essential amino acid, used in pharmaceuticals, in medical infusion solutions, and in special diet foodstuffs. Japan, primarily Ajinomoto and Kyowa Hakko, produces about two-thirds of the world volume of amino acids (31).

The amount of amino acids sold in Japan in 1977

Amino acid	Tons
Mono sodium glutamate	76,000
DL- or L-methionine	5,500
Glycine	2,700
L-Lysine	2,500-3,000
DL- and L-alanine	600
L-aspartic acid	540
L-phenylalanine	90
DL- and L-threonine	40
L-tryptophan	40
L-valine	13
L-isoleucine	11

Source: ref. 26

66. L-lysine, L-tryptophan and L- or DL-methionine are the most common amino acid animal feed supplements. Their world-wide demand is also increasing in the health food industry, and in bio-research. A world-wide market survey of amino acid production showed that both the synthetic DL-methionine and its hydroxy analog used as feed supplements are both produced commercially from petrochemicals, not via fermentation. L-lysine and L-tryptophan, on the other hand, are usually produced by fermentation (33). In the early 1970's the only producers of L-lysine were the Japanese companies Ajinomoto and Bio-Kyowa. Since then, the South Korean firm Miwon, the French company Eurolysine and the Mexican company Fermex have joined the ranks. No American company currently produce L-lysine. In fact in the 1960's and early 1970's the only large American companies producing any amino acids were Du Pont, Monsanto, and Stauffer Chemical Co. Stauffer closed its monosodium glutamate plant in 1983 because of severe competition from foreign suppliers (33).

67. Producers of L- or DL-tryptophan on the industrial scale include Japanese companies Ajinomoto, Kyowa Hakko, Tanabe Seiyaku, Mitsui Toatsu, and Showa Denko. In Europe, tryptophan is produced by Degussa only in semi-commercial quantities. In the U.S.A., there are no companies producing tryptophan (33). In 1983 two Japanese firms announced plans to produce L-lysine at plants in the U.S.A.: Bio-Kyowa, in Cape Giradeau, Missouri, with the projected yearly plant capacity of 15,000 tons, and Ajinomoto, in Eddyville, Iowa, with a yearly production capacity of 6,000 tons. The current U.S. demand in L-lysine is 24,000 tons/year. American producers sell lysine at the current market price of \$ 1.40/pound (\$ 3.11/kg), and L-tryptophan at almost \$ 9/pound (\$ 20/kg) (34).

68. The data indicated in the above paragraph refer mainly to the microbiological processes of producing amino acids. During the last two decades, however, a new approach to the production of the optically active amino acids has been developing, using immobilized aminoacylase, or L-amino acid acylase. This was the first industrial application of immobilized enzymes, occurring in 1969 when the Tanabe Seiyaku Company, Ltd., of Japan, initiated its process for producing L-methionine.

A. A background

69. Chemical synthesis of amino acids generally produce an optically inactive racemic mixture, i.e. both the L- and D-isomers are produced. To obtain natural L-amino acid from the chemically synthesized DL-form, optical resolution is necessary. Among the many optical resolution methods, the enzymatic method using microbial aminoacylase is one of the most advantageous procedures, yielding optically pure L-amino acids. The enzyme is specific for the L-form and thus a chemically synthesized acyl-DL-amino acid is asymmetrically hydrolysed by aminoacylase to give L-amino acid and unhydrolyzed acyl-D-amino acid. Both products are easily separated by their differing charge and solubilities. Acyl-D-amino acid is then racemized by heat treatment into a racemic mixture of acyl-D- and acyl-L-amino acids, and reused for the resolution procedure. Because mold aminoacylase has a broad substrate specificity and attacks acyl-L-amino acids with various side-chains, the enzymatic optical resolution of racemates can be applied to various amino acids (35).

70. From 1954 to 1969, this enzymatic resolution method has been employed by Tanabe Seiyaku Co. Ltd. using soluble **Aspergillus oryzae** aminoacylase for the industrial production of several L-amino acids. The enzyme reaction was carried out batchwise. This procedure, however, had some disadvantages inherent in a batch process using soluble enzyme; for instance, in order to isolate an L-amino acid from the reaction mixture, it was necessary to remove enzyme protein by pH and/or heat treatments (36). Such a step resulted in uneconomical use of enzyme, the yield of L-amino acid was lowered, and a great deal of labour was necessary for batch operation. Therefore, as a result of extensive studies of the continuous optical resolution of DL-amino acids using immobilized aminoacylase, the industrial production of L-amino acids was switched over to the immobilized enzyme process. This first occurred in 1969 with the production of L-methionine.

B. Commercial preparations of immobilized aminoacylase

71. As with immobilized glucose isomerase (see paragraphs 23-33), among preparations of immobilized aminoacylase intended for an industrial application there are not many covalently bonded enzymes. The best known preparations include aminoacylase immobilized by ionic binding to DEAE-Sephadex (developed by Tanabe Seiyaku Co.), and the enzyme entrapped as microdroplets of its aqueous solution into fibres of cellulose triacitate by means of fibre wet spinning (developed by Snam Progetti, see also paragraph 25). Tanabe indicates that the preparation of its immobilized enzyme is easy, the activity is "stable and high", and the regeneration of deteriorated preparation is possible. The last point is important because DEAE-Sephadex is a very expensive carrier. The immobilized enzyme is prepared as follows. 1,000 l of DEAE Sephadex A-25 and 1,100-1,700 l of aqueous solution of aminoacylase are mixed and stirred at 35°C and pH 7.0 for 10 hr, then filtered and washed with water. The yield of active enzyme in relation to the initial enzyme preparation is 50-60%. The half-life time of the DEAE-Sephadex aminoacylase is equal to 65 days at 50°C, in comparison with 48 days at 37°C for aminoacylase entrapped in polyacrylamide gel (36). According to Snam Progetti's data (12), the entrapped preparations of aminoacylase from hog kidney and microorganisms have shown "very good" stability in operating conditions in the course of resolving racemic mixtures of N-acetylmethionine; the loss of the enzyme activity was only 25-30% after 50 days of operation.

72. Rohm GmbH (Darmstadt, FRG) has used for immobilization of amino acid acylase a macroporous bead made of plexiglas-like material, a carrier with a porosity of 3-4 mg/g, onto which the enzyme was bound covalently by oxirane groups. Because of their rigid structure the 0.1-0.3 mm diameter beads are pressure stable and show "good flow properties" (37). The immobilized aminoacylase has a brand name Plexazym AC, and 1 g of it at operating conditions was as active as 0.4 g of the original purchased enzyme preparation (37).

C. Technological characteristics of the processes

73. The flow diagram for the continuous producing of L-amino acids by Tanabe Seiyaku Co. is shown in Figure 7. Substrate, that is N-acetyl-DL-amino acid solution, is continuously pumped into an enzyme column at a constant flow rate through a filter and heat exchanger by chemical pump. As it passes through the column, the substrate is converted to L-amino acid and N-acetyl-D-amino acid. Enzyme column effluent is concentrated, and L-amino acid is crystallized. Acetyl D-amino acid contained in mother liquor is racemized by heating in a racemization tank, and reused for optical resolution. The system is automatically controlled and operated continuously. The reaction takes place at pH 7.0 (in the presence of 5.10^{-4}M cobalt salts), 50°C; the flow velocity (in a downflow operation) is 900-20,000 l/hr. The volume of the column is 1 m^3.

74. The aminoacylase column maintained about 70% of the initial activity after 30 days of operation, and the half-life of the enzyme in the column was estimated to be about 65 days. The ratio of height: diameter did not influence the process efficiency. A deteriorated column is completely reactivated simply by the addition of the amount of aminoacylase corresponding to the deteriorated activity. The stability of the water insoluble carrier DEAE-Sephadex is very high, and according to Tanabe Seiyaku (36) it has been used for over 8 years without significant loss of binding capacity or physical decomposition.

75. As an example, continuous production of L-methionine using a 1000-litre enzyme column was as follows (38). A solution of 0.2 M acetyl-DL-methionine (pH 7.0, $5x10^{-4}$M Co^{++}) was passed through the aminoacylase column at a flow rate of 2000 l/hr at 50°C; 2000 litres of the effluent were evaporated, and the separated crude L-methionine was collected by centrifugation and recrystallized from water. The yield was 27 kg (91% of the theoretical). The residual acetyl-D-methionine in the mother liquor was heated at 60°C with acetic anhydride for racemization. The reaction mixture was adjusted to pH 1.8 and the separated acetyl-DL-methionine was collected and reused as substrate. The yield was 36 kg (94% of the theoretical).

76. Since 1969 Tanabe Seiyaku has been industrially operating several series of enzyme reactors in the company's plants for the production of L-methionine, L-valine, L-phenylalanine and some other L-amino acids.

Optical resolution of N-acetyl-DL-amino acids by aminoacylase immobilized on DEAE-Sephadex and using a 1,000 l reactor of Tanabe Seiyaku Co.

Amino acid	Flow velocity (volume/hr)	Yield of amino acids (kg) 24 hr	30 days
L-alanine	1.0	214	6420
L-methionine	2.0	715	21450
L-phenylalanine	1.5	594	17820
L-tryptophan	0.9	441	13230
L-valine	1.8	505	15150

Source: ref. 38

77. A basically similar approach has been applied by Snam Progetti (Italy) for a batchwise resolution of a racemic mixture of N-acetyl-DL-tryptophan using a small pilot plant (12, 39). Aminoacid acylase entrapped in cellulose triacetate fibers has been used to produce L-tryptophan and N-acetyl-D-tryptophan. In the pilot process the feed tank contained 9.85 kg acetyl-DL-tryptophan, 23.8 g $CoCl_2.6H_2O$, 1.6 kg NaOH (for pH 7.0) and 200 l water at 45°C. This solution was recycled for 5.5 hr (after which the hydrolysis was practically complete) through the enzyme reactor at a flow velocity of 350 l/hr. The reactor was 77 cm high, 19 cm in diameter, with 4 kg of dry fibre, containing 0.28 kg protein/kg dry fibre. The hydrolyzed material was then evaporated under vacuum to 15 l and separated, based on solubility differences, to give 3.9 kg of L-tryptophan (95% yield) and 15 l of acetyl D-tryptophan solution. The latter was mixed with 3 l of acetic anhydride and held for 5 hr at 45°C to give 4.7 kg of racemized and precipitated product (yield 96%). The racemized acetyl-D-tryptophan was then recycled to the feed tank. The small pilot plant, having a capacity of about 1 kg of L-tryptophan/hr, has been operating for several months. The activity of the aminoacylase fibres showed a 20% decrease during this period, and productivity was about 400 kg of L-tryptophan per kilogram of fibre. The same approach has been used also for the resolution of N-acetyl derivatives of DL-valine and DL-methionine (12).

78. Degussa AG (West Germany) reported in 1983 that it has a plan to develop by 1985-1988 a new technology for producing optically and chemically pure L-amino acids, namely arginine, isoleucine, threonine, proline, and tryptophan (40). The company does not use immobilized enzymes for the optical resolution of racemates but uses instead a membrane reactor fed with the soluble catalyst, aminoacylase. A Degussa-produced N-acetyl racemic amino acid solution is cycled through the membrane reactor where acylase, produced by Amano Pharmaceutical Co., Ltd. in Nagoya, Japan, produces deacylated L-amino acids. The latter, of lower molecular weight as compared with N-acetyl-D- and N-acetyl-L-amino acids, passes through the reactor's membrane. The enzyme, because of its high molecular weight, is also retained in the vessel (41). Separated L-amino acids are collected by ion exchange or crystallization. After racemization, the remaining solution is recycled with more of the original N-acetyl-DL-amino acid substrate. According to a Degussa representative, the continuous-production process using the membrane reactor is more efficient than other methods, including those using carrier-fixed enzymes (41).

79. Another process developed by Degussa which became the first industrial application of a two-enzyme system with cofactor regeneration was the production of some L-amino acids from cheap keto-acids. The regeneration of cofactors like NAD or ATP, which dissociate from their apoenzymes, is a serious problem in enzyme engineering, because dissociating coenzymes must retain a degree of mobility in order to have access to the active centres of two apoenzymes. The alternative, i.e. continuous replacement of the cofactor, would be highly uneconomical. In the Degussa process, NAD^+ is coupled to polyethylene glycol and retained in the membrane reactor with keto acid dehydrogenase and formate dehydrogenase (42).

80. In a Rohm GmbH pilot process, N-acetyl-DL-amino acids were converted to L-amino acids in a packed bed reactor at 37°C by Plexazym AC (see paragraph 72). N-acetyl-DL-methionine, 0.7 M at pH 8, was 80% hydrolyzed at a space velocity of 6 hr^{-1}. The rate of hydrolysis decreased to about 30% when the space velocity was increased to 25 hr^{-1}. A productivity of 500 kg L-methionine/kg Plexazym AC was reached in a 90 days reactor run (37).

81. Recently, Tanabe Seiyaku has announced that a new approach for the continuous production of L-alanine from L-aspartic acid developed by the company was under investigation (43). In that approach, immobilized Pseudomonas dacunhae cells with high L-aspartate beta-decarboxylase activity are used. The decarboxylase enzyme shows high enantiomer selectivity reacting only with L-aspartic acid. Thus, L-alanine and D-aspartic acid can be produced from LD-aspartic acid at the same time. Meanwhile, D-aspartic acid is used as an important intermediate for semi-synthetic penicillin, the synthesis of which has been also developed by Tanabe Seiyaku Co. As the company reported, however, in this continuous decarboxylase system there are problems associated with evolution of CO_2 gas during the reaction. It is difficult to maintain the plug-flow of the substrate solution under normal pressure, and to keep a constant pH of reaction mixture in the reactor because of the CO_2 effervescence. The company therefore designed a closed column reactor which performs the enzyme reaction at an elevated pressure such as 10 kg/cm^2. Since liberated CO_2 gas mixes into the reaction mixture, the complete plug-flow of the substrate solution is maintained and the pH of the reaction mixture is not appreciably changed. Moreover, the efficiency of immobilized cells for producing L-alanine in the closed column system at high pressure increased by 1.5 times (from 250 to 360 mmole/l/hr) as compared with that at the normal pressure conditions; the stability of the immobilized cells is not affected by pressure elevation (43). It was reported (44) that Tanabe Seiyaku established the continuous production process for L-alanine in 1982 and succeeded in its commercial production.

D. Economic estimates

82. A comparison of the cost for production of L-amino acids by soluble and immobilized aminoacid acylase is shown in Figure 8. With the immobilized enzyme, the purification procedure for product became simpler and the yield was higher than in the case of the soluble enzyme. Less substrate was required for the production of a unit amount of L-amino acid. Because the immobilized aminoacylase was stable, the cost of enzyme was markedly reduced compared with that of enzyme in the soluble system. In the case of immobilized enzyme, the process was automatically controlled, and the labour cost was also substantially reduced. As a result, the overall production cost of the immobilized enzyme process was about 60 % of that of the conventional batch process which uses soluble enzyme (36). It should be noted that the cost of the enzyme in this process is only approximately 1-2 per cent of the overall operating budget, as was estimated in 1984 (5).

E. Scale of the processes

83. By 1971 the reported capacity of the Tanabe Seiyaku' process (using immobilized aminoacylase) was greater than 700 kg of L-amino acids per day (45). Immobilized aminoacylase columns usually produce up to 750 kg of product per day (5). According to 1984 data, this enzyme technology is currently used to produce over 50,000 tons of L-amino acids annually (5). Contrary to this, in another publication in 1984 (29) it was reported that presumably less than 250 tons of L-amino acids is produced by this technology per year, and the estimated immobilized enzyme amount is less than 1 ton/year. Amano (Japan) also uses immobilized aminoacylase in industry, as reported in (29).

84. In 1981 Degussa installed an experimental 5-ton-a-year plant in Konstanz, West Germany, producing L-amino acids: alanine, methionine, valine, phenylalanine, and tryptophan. In 1982 the production volume of the plant increased up to 60 ton/year as a result of the new method of separating amino acids from protein hydrolysates by means of ion exchangers. In 1984 the company planned to install at Valencia de Don Juan, Spain, a new 6,000 ton/year plant for L-lysine production by fermentation method (40,41), and has produced 10-15 tons per month of L-methionine, L-valine and L-phenylalanine by means of the two-enzyme system with cofactor regeneration (42) (see par.79).

III. PRODUCTION OF OPTICALLY ACTIVE D-AMINO ACIDS BY IMMOBILIZED HYDANTOINASE

85. In 1976-1977 Snamprogetti, the engineering company of ENI group, has developed an original process for the production of optically active D-amino acids, particularly D (-)-phenylglycine, which serves as an important intermediate for the industrial production of semisynthetic penicillins and cephalosporins. The method starts from the racemic amino acid hydantoins, which are hydrolyzed stereospecifically by the immobilized hydantoinase to the corresponding carbamoyl derivatives and finally transformed into the optically active amino acids.

A. Enzymes

86. Snamprogetti has discovered on enzyme, named hydantoinase, capable of opening the ring of 5'-substituted hydatoins. Hydantoinases have been extracted from different sources, both microbial and animal, and some of them are strictly specific for the D-hydantoins, others hydrolyse only the L-form (12).

B. A Background

87. Hydantoins which are intermediates in chemical synthesis of several amino acids, can be easily prepared by reaction of corresponding aldehydes with potassium cyanide and ammonium carbonate (46). The raw materials are generally cheap and the yield is high. The big advantage of hydantoins that form a basis for their application in enzyme engineering is that they undergo spontaneous racemization very easily and under mild conditions. As a result, two reactions occur simultaneously in the reaction mixture containing DL-hydantion and the enzyme hydantoinase: enzymic ring-opening in D-hydantoin (with the formation of corresponding D-carbamoyl derivative) and the

chemical racemization of the residual L-hydantoin. Thus, the whole amount of the initial racemic DL-hydantoin is rapidly transformed into the D-carbamoyl derivative, which in turn can be hydrolyzed chemically to the corresponding amino acid under conditions where complete retention of configuration is achieved (at 100°C in the presence of calcium hydroxide).

Rates of chemical racemization and the enzymatic hydrolysis of several hydantoins

Amino acid hydantoin	Half-life (min)	Relative rate of hydrolysis (the calf liver enzyme)
alpha-Phenylglycine	19	100
p-OH-Phenylglycine	40	3
Methionine	98	20
Alanine	262	32
Glutamic acid	327	0
Valine	1195	12

Source: ref. 12

88. Hydantoinase from calf liver and specific for the D-hydantoins has been chosen by Snamprogetti as the principal catalyst for immobilization and scaling-up the process. The enzyme preparation having a specific activity of about 3 U/mg protein was entrapped in cellulose triacetate fibers (paragraph 25). 70 U were entrapped per gram of polymer and about 80 % of this amount was found active in fibres (46).

C. Technological characteristics of the process

89. A stirred tank reactor for the hydrolysis of 3 kg of 5'-phenylhydantoin per day (the reaction actually takes place during 5 hr) has been described (12). The operating conditions were 30°C, pH 8.5; the half-life of the immobilized enzyme was 20 days. A similar approach was used also for the preparation of another intermediate for semisynthetic antibiotics, i.e. D(-)-p-hydroxyphenylglycine. As Snamprogetti reported (47), a method has been developed using an ion exchange resin, which removes the produced D-amino acid from the reaction mixture. The removal prevents oxidation of the NH^2 of the amino acid by nitrous acid present for the oxidation reactions.

D. Scale of the process

90. In 1977 Snamprogetti planned to install the plant with a capacity of 200 kg of D-phenylglycine per day (60 ton/year). Production was to be done batchwise, with the operation over 24 hours (47); one synthesis of phenyl hydantoin, one enzymic hydrolysis to carbamoyl derivative and 8 oxidation reactions were to be performed per day. At present, presumably less than 50 tons of D-phenylglycine is produced per year, with the estimated enzyme amount less than 1 ton/year (29).

91. The following items have been named as necessary equipment (47): one autoclave for hydantoin synthesis with a nominal capacity of 5 m^3; four stirred thermostated vessels for enzymic hydrolysis with a nominal capacity of 6.5 m^3 each; one filter press with a filter area of 5.0 sq.m; three column reactors for chemical oxidation of carbamoyl derivative with a nominal capacity of 3 m^3 each; three centrifugal pumps for the reactors with a capacity of 10 m^3/hr; one vacuum evaporator with a capacity of 500 kg water/hr, and maximum temperature of 50°C; one centrifugal separator with a basket capacity of 50 kg; one vacuum dryer for maximum temperature of 50°C.

92. More recent data about this process indicated (48) that the production of D(-)-p-hydroxyphenylglycine (an intermediate in the synthesis of semisynthetic penicillins and cephalosporins, especially amoxicillin) from its hydantoin precursor by hydantoinase was to be operated industrially by Kanegafuchi Chemical Industry Co. in Japan. In 1982 there was a report on the setting-up of an industrial plant in Singapore to produce the compound using immobilized Bacillus brevis cells containing enzyme dihydropyrimidinase, on the basis of the patented process by Kanegafuchi [cit.in (49)]. The plant has a production capacity of less than 50 tons per annum. A yield of 300 tons in 1983 and a rapid further increase to 700 tons per year [ibid.] was reported.

IV. PRODUCTION OF L-ASPARTIC ACID BY IMMOBILIZED MICROBIAL CELLS CONTAINING ASPARTASE

93. L-Aspartic acid is widely used for medicines and food additives and it has been industrially produced by fermentative and enzymatic methods from ammonium fumarate using the action of aspartase. There were some disadvantages, as this reaction was carried out on the industrial scale in batch process using soluble enzyme, similar to the case of soluble amino acylase process (paragraph 70). As a result of extensive studies into continuous production of L-aspartic acid using the immobilized Escherichia coli cells containing aspartase, Tanabe Seiyaku Co. industrialized the method in 1973. Later, in 1976, this process was also scaled up by Kyowa Hakko Kogyo Co. which has, since then, been using aspartase, immobilized on a polymeric carrier.

A. A background

94. The enzyme aspartase catalyzes a one-step stereospecific addition of ammonia to the double bond of fumaric acid. Fumaric acid is dissolved in a 25 % ammonia solution and the ammonium fumarate is passed through the reactor containing the enzyme catalyst. The reaction is exothermic and the reactor has to be designed to remove the heat produced.

95. Because aspartase is an intracellular enzyme it is necessary to extract the enzyme from microbial cells before immobilization. Extracted intracellular enzymes are generally unstable, and most of the immobilization methods which Tanabe Seiyaku tried resulted in low activity and poor yield (35). The company indicated that although entrappment of the soluble aspartase into a polyacrylamide gel lattice gave relatively active immobilized enzyme, its operational stability was not sufficient (see paragraph 97).

Therefore, Tanabe Seiyaku considered immobilized aspartase as unsatisfactory for the industrial production of L-aspartic acid. As a result of further studies, the company chose the direct immobilization of whole microbial cells of E.coli by entrapping them in polyacrylamide gel, and this system has been operating industrially by Tanabe Seiyaku Co, since 1973, in their plant for automatic and continuous production of L-aspartic acid. This is considered to be the first industrial application of immobilized microbial cells (35).

B. Immobilized enzymes (cells) preparations

96. Tanabe Seiyaku uses the enzyme aspartase from E.coli. After entrappment the cells are incubated in 1 mM Mg^{++} which causes autolysis of the entrapped cells and increases the effective activity greatly (7). In another report (36), when immobilized E.coli cells were suspended at 37°C for 24-48 hours in substrate solution, their activity increased by a factor 10. The increase of enzyme activity was observed even in the presence of chloramphenicol, inhibitor of protein synthesis. Therefore, this increase in activity was considered not to be the result of protein synthesis but due to increased permeability for substrate and/or product caused by autolysis of E.coli cells in the gel lattice. Autolysis was confirmed by electron micrographs of immobilized E.coli cells. After the lysis occurred the aspartase could not leak out of the gel lattice, even though the substrate, ammonium fumarate, and the product, L-aspartate, passed easily through the gel (36).

97. The immobilized catalyst is prepared as follows (35). Escherichia coli cells (100 kg, wet weight) collected from culture broth are suspended in 400 litres of physiological saline. To this suspension 75 kg of acrylamide monomer, 4 kg of N,N' -methylene-bis-acrylamide, 50 litre of 5% /a/-dimethylaminopropionitrile, and 50 litre of 1% potassium persulfate are added. The mixture is allowed to stand at a temperature below 40°C for 10-15 min, and the resulting stiff gel is made into round 2-3 mm granules. The half-life time for this preparation at 37°C was estimated to be 120 days. This can be compared with the half-life for the gel entrapped extracellular aspartase being equal to 30 days, and for the intact E.coli cells, which is 10 days.

98. As Tanabe Seiyaki has indicated, immobilization of enzymes and microbial cells into polyacrylamide gel has some limitations. Aspartase, for example, is partially inactivated during the immobilization procedure by the action of acrylamide monomer, beta-dimethylaminopropionitrile or by potassium persulfate. By way of a further improvement in the productivities of immobilized microbial cell systems, the company has found that kappa-carrageenan, a polysaccharide from seaweeds, was one of the most suitable matrices for immobilization of enzymes and microbial cells (35). Kappa-carrageenan easily becomes a gel under mild conditions and does not inactivate enzymes in the course of their immobilization. Thus, E.coli immobilized with the carrageenan and then treated with glutaraldehyde and hexamethylenediamine shows the highest aspartase productivity. When the productivity of immobilized preparation with polyacrylamide was taken as 100, that of immobilized cells with carrageenan hardened with glutaraldehyde and

hexamethylenediamine was 1500 - around 15 times greater. The last preparation was also very stable, with a half-life of 680 days (almost 2 years). As a result, in 1978 Tanabe Seiyaku Co. switched from the conventional polyacrylamide method to the new carrageenan method for industrial production of L-aspartic acid.

Immobilized Escherichia coli cells producing L-aspartic acid

Immobilization method	Asparatase activity (units/g cells)	Stability at 37°C (half-life,day)	Relative productivity
Polyacrylamide	18,850	120	100
Carrageenan	56,340	70	174
Carragenan + glutaraldehyde (GA)	37,460	240	400
Carrageenan +GA + hexamethylenediamine	49,400	680	1500

Source: ref. 35

99. After examining several adsorbents the Kyowa Hakko Kogyo Co. selected Duolite A^7, a phenol-formaldehyde resin for adsorbing aspartase used in their continuous production of L-aspartic acid with a packed bed reactor (50).

C. Technological characteristics of the processes

100. Ammonium fumarate serves as the principal substrate for the preparation of L-asparatic acid using immobilized aspartase (see also paragraph 94). The flowsheet of the process is essentially the same as that for the optical resolution of amino acid racemates (Figure 7). That is, 1 mole/l solution of ammonium fumarate containing 0.001 mole/l $MgCl_2$ is passed through the column packed with the immobilized E.coli cells at 37°C and pH 8.5. The flow velocity is equal to 0.6 volume/hr. In a typical run the 2400 litre effluent is adjusted to pH 2.8 with 60% sulfuric acid at 90°C, then cooled to 15°C and kept for 2 hr. Crystallizing L-aspartic acid is collected by centrifugation and washed. By this simple procedure pure L-aspartic acid can be obtained without recrystallization, and the yield is usually over 95% (35). The industrial process is a fully automatic and continuous one.

101. Japan's Mitsubishi Petrochemical Co. announced (51) that it would begin, in the fall of 1986, making L-aspartic acid from fumaric acid and ammonia using the microorganism aspartase containing **Brevibacterium flavum**. **E.coli** is usually used but has a weak cell structure that must be protected by immobilizing the bacterium if the microorganism is to be recycled. B.flavum, on the other hand, has a hard cell wall that can withstand repeated centrifugations without a need for immobilization, thereby reducing the cost of L-aspartic acid production.

D. Economic estimations

102. A comparison of the cost of production of L-aspartic acid by the conventional batch process using intact cells and the continuous process using immobilized cells is shown in Figure 9. The major savings are in the cost of the enzyme which is reduced by a factor of approximately 9 because immobilized cells are substantially more stable as compared with intact cells. There is also a 30% reduction in labour cost since the immobilized process is automated. As a result, the overall production costs of the immobilized cell system are about 60% that of the conventional batch process using intact cells. Furthermore, the procedure employing immobilized cells is advantageous from the standpoint of waste treatment (35).

E. Scale of the processes

103. Tanabe Seiyaku Co. uses a 1000-liter column with immobilized cells and produces 1700 kg/day (89% of the theoretical yield) or 51 ton/month of L-aspartic acid (35). A similar process has been operating industrially also by Kyowa Hakko Kogyo Co. since 1976.

104. In the fall of 1986, Mitsubishi Petrochemical Co. expected to begin to operate a plant producing L-aspartic acid using **B.flavum** aspartase (see paragraph 101) with a capacity of 1,000 tons per year (51).

105. Purification Engineering, Inc. (Baltimore, MD) recently claimed that it can make some 2,000 tons of L-aspartic acid a year - almost as much as required for all of G.D.Searle's (Skokie, IL) $ 500 million aspartame production - in a 208-liter immobilized-cell column. The $4 million plant producing L-aspartic acid and L-phenylalanine has been on-line since December 1984 (52).

V. PRODUCTION OF L-MALIC ACID BY IMMOBILIZED MICROBIAL CELLS CONTAINING FUMARASE

106. L-Malic acid is mainly used in the pharmaceutical field as an antidote for hyper-ammoniemia and as a component of aminoacid infusion. It is becoming of greater market interest as food acidulant in competition with citric acid, for example, in confectionery products. Malic acid, which is found naturally in most fruits, is a weaker acid than citric or tartaric. It possesses a tart flavour which builds up slowly and then gradually diminishes, blending well with essences and other flavours. The racemic mixture of DL-malic acid is produced by a cheap chemical synthesis from malic anhydride and water, whereas the natural L-form of malic acid produced by fermentation in batch process using microbial broth is too expensive to open its own market in the field of the acidulants.

107. The continuous industrial production of L-malic acid by means of immobilized microbial cells **Brevibacterium ammoniagenes** followed by **B.flavum** containing the enzyme fumarase has been operating by Tanabe Seiyaku Co. since 1974 (36). Some time later a process for the enzymic hydration of fumaric acid to L-malic acid by fibre-entrapped fumarase. This was recommended to an industrial operation (12) previously extracted from microbial cells and purified. In 1976, similar process was industrialized by Kyowa Hakko using fumarase adsorbed on an anion exchange resin (50).

A. A background

108. L-Malic acid is produced industrially as a result of hydration of fumaric acid by the enzyme fumarase, either intracellular or extracted from a microbial producer. The reaction reaches an equilibrium when about 80% of the fumaric acid is converted to L-malic acid.

109. According to Tanabe Seiyaku (53) immobilized cells B.ammoniagenes form succinic acid as a by-product along with L-malic acid. Separation of these two products is very difficult. Therefore, the critical point of success for industrial production of pure L-malic acid is prevention of succinic acid formation during the enzyme reaction. Treatment of the immobilized cells with detergents, particularly with readily available bile extract, was considered by Tanabe as most suitable for industrial purposes. The detergent suppresses succinic acid formation, and greatly enhances the formation of L-malic acid by the immobilized cells. The most effective conditions were found as follows: Immobilized cells are incubated in 1 M sodium fumarate (pH 7.5) containing 0.3% bile extract at 37°C for 20 hr (53).

B. Immobilized cells and enzymes

110. Tanabe Seiyaku used fumarase-containing cells **Brevibacterium ammoniagenes** immobilized in polyacrylamide gel (35,36) during the first three years (1974-1977) of industrial production of L-malic acid. This led to a better stability of the enzymic catalyst as compared with catalysis using intact cells. Thus, the activity of intact cells in the operating conditions (37°C) rapidly decreased and its half-life amounted to 6 days. On the other hand, immobilized cells treated with bile extract had a half-life of 55 days at the same temperature.

111. Tanabe Seiyaku, however, has indicated that immobilization of microbial cells by polyacrylamide gel has some limitations. That is, some enzyme molecules were inactivated during the immobilization procedure by the action of acrylamide monomer, betadimethylaminopropionitril, potassium persulfate or heat of the polymerization reaction (see also paragraph 98). So in order to find a more general immobilization technique and to improve the productivities of the immobilized microbial cell system the company switched over to kappa-carrageenan as a gel matrix (par. 98). In addition, the company discovered that **Brevibacterium flavum** has a higher fumarase activity and stability after immobilization with carraggenan as compared with the former B.ammoniagenes (35).

112. When the productivity of immobilized **B.ammoniagenes** with polyacrylamide was taken as 100, that of immobilized **B.flavum** with the same carrier was 273 and for **B.flavum** with carrageenan was 897. The half-life time at 37°C for the immobilized microbial systems is as follows:

B.ammoniagenes	
Polyacrylamide	53 days
Carrageenan	75 days
B.flavum	
Polyacrylamide	94 days
Carrageenan	160 days

113. For immobilization both cell suspension and carrageenan dissolved in physiological saline are warmed to 37-60°C. Both are mixed, and the mixture is cooled and/or contacted with aqueous solution containing K^+, NH_4^+, Ca^{++}, or other ions as a gel-inducing agent. After treatment, the gel is granulated to a suitable particle size. If the operational stability of immobilized cells is not satisfactory, they can be treated with hardening bifunctional agents such as glutaraldehyde and hexamethylenediamine to obtain stable immobilized cell preparations (35).

114. In order to obtain even more stable preparations and to improve the productivity of immobilized fumarase cells, Tanabe Seiyaku Co. at the end of the 1970's started to add polycationic polymers to the immobilization medium. The stabilization effect was particularly evident with polyethyleneimine (54). The heat stability of the immobilized preparation increased so that the column could be operated at relatively high temperatures of 50-55°C for long periods (half-life 128 days at 50°C, 74 days at 55°C and 243 days at 37°C). The productivity of **B.flavum** immobilized with kappa-carrageenan and polyethyleneimine increased to 21 times that of **B.ammoniagenes** immobilized with polyacrylamide (2100 at 45°C and 1600 at 37°C as compared with the data in paragraph 112). In 1980 the industrial production system of L-malic acid was changed using this immobilization method.

115. According to the Snamprogetti's procedure (12), fumarase was extracted and partially purified from microbial cells by mechanical disruption, removal of nucleic acids, and precipitation with ammonium sulfate. The purified enzyme solution was entrapped in cellulose triacetate fibres which displayed about 40% of the entrapped activity. The characteristics of the immobilized enzyme have not been disclosed, and according to (12), the entrapped fumarase showed "good" stability both under storage and operating conditions at pH values of 7.0-8.0, as compared with the free enzyme which was "rather unstable", especially when dissolved in water at a low protein concentration.

C. Technological characteristics of the processes

116. Tanabe Seiyaku uses a column packed with immobilized cells. The inlet solution of 1 M sodium fumarate at pH 7.0 is passed through the column at 37°C at flow rate 0.2 hr^{-1}; the reaction to these conditions reaches an equilibrium with about 80% conversion of fumaric acid to L-malic acid. From the effluent of the column, L-malic acid is obtained by ordinary methods, that is, fumaric acid is separated by acidifying the effluent. L-malic acid is then separated with about 70% yield from raw material, fumaric acid (53).

117. In the Snamprogetti process, a solution of 0.5 M sodium fumarate at pH 7.0 and 25°C is continuously pumped through a column packed with fibre-entrapped fumarase. 50% of fumarate is converted to L-malate, and the decrease of the enzymatic activity is 25% of the initial value after 100 days of operation. L-Malic acid is separated from the reaction mixture by precipitation as calcium salt from which pure L-malic acid is obtained; the unchanged fumarate is recycled (12).

118. In China, L-malic acid is produced with 15% polyacrylamide gel immobilized **Candida rugosa** cells from fumarate in 82-85% yield in continuous column operation (18). As the author reported, "pilot plant experiments and industrial production [of L-malic acid] have been accomplished".

D. Economic estimation and scale of the processes

119. Economic estimates and data on scales of the processes of L-malic acid industrial production by means of immobilized enzymes or cells are generally lacking in the literature. Tanabe Seiyaku reported only, that "both the economic efficiency and the quality of the product have so far been satisfactory" (35). According to Snamprogetti, a comparison between the production cost of citric acid by fermentation and that of L-malic acid by fibre-entrapped fumarase "was done and gave comparable production costs for the two organic acids" (12).

120. According to (48), production of L-malic acid using immobilized microbial cells and enzyme is now industrially operated by Tanabe Pharmaceutical Co., and Kyowa Hakko Kogyo Co.

VI. DEACYLATION OF PENICILLIN AND CEPHALOSPORIN DERIVATIVES BY IMMOBILIZED PENICILLIN AMIDASE

121. Beta-lactam antibiotics make up a large group of physiologically active compounds including natural and semisynthetic penicillins, cephalosporins, and cephamycins. Many of them are characterized by unique antimicrobial properties, bacteriocidal action, and low toxicity and are widely used for practical applications in medicine. Because of their practical effectiveness, the synthesis and modification of ß-lactam antibiotics are important targets of enzyme engineering (55). The present world market value of antibiotics for pharmaceutical use is estimated annually at nearly 5 billion US $, and ß-lactams account for 53.5% (1980) and 63.5% (1990, expected) out of it (31).

122. Synthesis of new antibiotics usually proceeds in two major steps. For the first step a natural ß-lactam compound (e.g. penicillin or cephalosporin) is hydrolyzed by penicillin amidase forming a semiproduct such as 6-aminopenicillanic acid (6-APA) or 7-aminodeacetoxy-cephalosporanic acid (7-ADCA). For the second step the semiproducts form new "semisynthetic" antibiotics as result of chemical or enzymatic conversion.

123. Thus, 6-APA and 7-ADCA are important intermediates in the production of semisynthetic penicillins and cephalosporins in the pharmaceutical industry. Penicillins are well-known medical preparations and have been widely used since 1930's. Cephalosporins, on the other hand, have demonstrated some considerable advantage over penicillins in the broad spectrum of antimicrobial activity, low toxicity, and also outstanding efficacy against various penicillin resistant strains. 6-APA and 7-ADCA have been produced commercially for some years by the deacylation of benzylpenicillin (penicillin G) and a cephalosporin derivative respectively using immobilized penicillin amidase (penicillin acylase), the first industrial process for 6-APA production being realized in the beginning of 1970's by three manufacturers simultaneously: Squibb

(USA), Astra (Sweden), and Riga Biochemical Plant (USSR). 7-ADCA production using immobilized penicillin amidase was industrialized by Riga Biochemical Plant (USSR) in 1980.

A. A background

124. Penicillin amidase catalyzes the deacylation of penicillin with the formation of 6-APA and the side chain acid. There are several types of penicillin amidases and they are distinguished by their substrate specificity. Enzymes of one group, usually of fungal or of actinomycete origin preferentially hydrolyzes phenoxymethylpenicillin (penicillin V), whereas that of the other group, generally of bacterial origin, preferentially hydrolyzes benzylpenicillin (penicillin G). In the latter case the hydrolysis products are 6-APA and phenylacetic acid.

125. In contrast to most other industrial processes using immobilized cells, the deacylation of benzylpenicilein results in the formation of ionogenic compounds which at the normal operating pH are charged (at least one of them). To maintain the reaction pH in the desired range it is necessary therefore to add alkali or use large amounts of a buffering salt. This in turn affects the design and operation of immobilized penicillin amidase reactors.

126. Production of 6-APA by fermentation is uneconomical: yields are poor and are always accompanied by production of mixture of penicillins. The isolation of 6-APA, therefore, requires a complex process (56). The chemical method of 6-APA production from its respective penicillin usually depends upon the selective deacylation of penicillin while the ß-thiazolidine ring must be protected from opening. In addition, the method requires subzero temperatures. Moreover, problems of corrosion are severe because of aggressive solvents.

127. Before immobilized penicillin amidase was introduced commercially, 6-APA had been industrially produced batchwise using intact bacterial cells containing the enzyme. Cells were used only once and the wet microbial mass was usually discarded. Immobilization of penicillin amidase was allowed to run the process continuously. The enzymatic method for deacylation of penicillins has advantages over others as the hydrolysis proceeds to completion under mild conditions, for example, at temperatures slightly higher than ambient and at pH near neutrality. Under these conditions, both penicillin and 6-APA are stable and the product isolated is free from contamination of degraded compounds. Besides the evident economic advantages that immobilized systems offer as a result of their recycling potential, in the present context the need to exclude macromolecular allergenic impurities is another overiding reason for the exclusive use of the immobilized catalyst. Presently, the substantial part of 6-APA manufactured in Sweden and Italy, and all 6-APA produced in the USSR and in Japan is obtained using immobilized penicillin amidase.

128. In the overall process penicillin G is separated from the fermented broth by solvent extraction; the end product of the extraction is generally a crude concentrated solution of penicillin G from which the penicillin is crystallized by azotropic distillation with butanol. The dried crystals of penicillin G are dissolved in water and sent to the hydrolysis stage to yield 6-APA and phenyl acetic

acid. Certainly if the 6-APA plant is located adjacent to the penicillin fermentation plant, it would be possible to directly hydrolyze the crude solution of penicillin G obtained from the solvent extraction, by-passing the crystallization stage. The phenyl acetic acid formed can be used as a precursor in the penicillin fermentation (57).

B. Commercial preparations of immobilized penicillin amidase

129. A number of immobilized systems have been patented or commercially produced for penicillin amidase, which vary significantly (see the table below). The enzyme covalently coupled to Sephadex G 200 activated by cyanogen bromide is described in a patent (Ger. Offen. 1,961,428) assigned to the Swedish firm, Astra. Bayer A.G. have patented a series of hydrophilic supports covering polyacrylamide maleic acid copolymer prepared by heating at 80°C and maleic anhydride - polyol(meth)acrylate copolymer derivatives for the immobilization of penicillin amidase (Ger.Offen. 2,157,972; 2,215,509; 2,215,512; 2,215,687). Besides, Bayer A.G. patented the covalent attachment of the enzyme to soluble macromolecules like dextran or starch followed by encapsulation of the complex in a polymer (Ger.Offen. 2,313,,824). Novo Laboratory holds patents on enzyme immobilization by intermolecular cross-linking with bifunctional reagents like glutaraldehyde either in the presence of an inert filler like cellulose or by later admixture with the filler (Ger.Offen 2,345,185; 2,345,186). Cross-linking of the enzyme in a nonionic methacrylate resin support with glutaraldehyde added in the presence of a water soluble diamine has been described in a patent assigned to the Beecham group (Ger.Offen. 2,334,900; 2,336,829). Adsorption of the enzyme by ion exchangers like DEAE-cellulose and CM-cellulose and subsequent cross-linking with glutaraldehyde has also been patented by Beecham (Ger.Offen. 2,459,450). At Shamprogetti a partially purified penicillin amidase from **E.coli**, entrapped in spun fibres of cellulose triacetate has been successfully utilized on a laboratory scale since the beginning of 1970's (58) and then industrialized. Tanabe Seiyaku Co. holds a patent according to which whole cells of **E.coli** and other organisms containing the enzyme have been entrapped in polyacrylamide (Ger.Offen. 2,414,128). Several patents describe immobilized purified penicillin amidase from **E.coli** attached to cellulose derivatives such as DEAE cellulose, amino-chloro-S-triazyl DEAE cellulose, dichloro-S-triazyl DEAE cellulose, and filter cloth (S.African Pat. 6,804,164; 6,804,009; British Pat. 1,183,260). Toyo Jozo company has developed a process for the production of 7-ADCA in which the extracellular penicillin amidase adsorbed on celite or covalently coupled to activated porous fibres is used as catalyst (51). Spofa, United Pharmaceutical Works (Czechoslovakia) has been using covalently cross-linked disrupted microbial cells with a "high content" of penicillin amidase for commercial production of 6-APA (Czechoslov. Pat. 203,607).

Commercially available immobilized penicillin amidase preparations

Company	Immobilization procedure
Astra	Covalent coupling to Sepharose activated by cyanogen bromide
Bayer	Covalent coupling to a dextran activated by cyanogen bromide
Beecham	Cross-linking in a nonionic methacrylate resin with glutaraldehyde
Minmedbioprom (USSR)	Entrappment of enzyme in cross linked polyacrylamide gel; the thermostability is 10 times higher than that of native enzyme
Otsuka Pharmaceutical (Jap.Pat.7,228,183;7,228,187)	Covalent coupling of succinylated enzyme with DEAE-Sephadex
Snam Progetti	Entrappment in spun fibers of cellulose triacetate
Spofa (Czechoslovakia)	Covalent cross-linking of disrupted microbial cells
Squibb	Adsorption on bentonite
Tanabe Seiyaku Co.	Entrappment of whole cells in polyacrylamide gel
Toyo Jozo	a) Adsorption on celite b) Covalent coupling to activated porous fibres
Rohm GmbH	Covalent coupling to Eupergit, a macroporous beaded polymer

130. In a Chinese industrial process (18) an E.coli strain having"high penicillin acylase activity" is immobilized in agar gel. The microbial cells were mixed with an equal volume of 8% agar gel and poured into an organic solvent with stirring. After washing, the resulted gel beads were cross-linked with 1% glutaraldehyde solution and washed. Another immobilized preparation was obtained by entrappment of the microbial cells in gelatin and cross-linked with glutaraldehyde, then layered and cut into 2 mm gel film particles. According to (18,59) both immobilized preparations have been successfully used in the pharmaceutical industry since 1978 to produce 6-APA, and later, 7-ADCA.

131. As an alternative to the use of preformed polymers, Boehringer Mannheim has developed a method for immobilization of penicillin amidase in which the enzyme is derived from epoxides bearing a vinyl group, the soluble protein derivative being subsequently copolymerized with materials like acrylamide as carriers. The size of the particles and their physical properties have a bearing not only on agitation in the reactor but also on the separation of the catalyst by sedimentation or filtration (50).

132. Immobilization of penicillin amidase by the Snamprogetti's method is as follows (57). 10 kg of a solution of the enzyme containing glycerol, which has been phosphate buffered at pH 8, is added to a solution of 5 kg cellulose triacetate in 71.4 l methylene chloride at 4°C with stirring. The emulsion formed is extruded through a spinnerette into a coagulation bath containing toluene. The fibres formed are then dried to remove all organic solvents. Penicillin amidase as well as other proteins present in the enzyme preparation are physically entrapped within the microcavities of the porous fibres, 20-30 % of the initial enzyme activity being expressed by the fibres. One kg of dry fibres produces about 240 kg of 6-APA for a period of more than 4 months (60).

133. Genin, a Mexican company, announced in 1982 that it was going to make immobilized penicillin amidase by entrappment of genetically engineered **E.coli** that overproduced the enzyme, in kappa-carrageenan gel. The method avoided disrupting the E.coli cells to extract the amidase. A leader of the research project announced that the **E.coli** strain was the subject of an international patent application (61).

134. Rohm GmbH (F.R.Germany) has developed "EUPERGIT - Penicillin-amidase', that is penicillin amidase from E.coli, covalently bonded to EUPERGIT-C which is a macroporous beaded polymer composed of methacrylamide, N-methylene-bis-methacrylamide and allylglycidyl-ether (62). The beaded shape of the catalyst and its mechanical rigidity allows easy filtration: emptying times of about 15-45 min for 1500 liters from a batch reactor have been observed (62).

C. Technological characteristics of processes

135. Toyo Jozo used immobilized penicillin amidase in a column reactor composed of six layers or packs of the enzyme-bed. Deteriorated enzyme packs are taken off one by one and new active packs are added on. After about every 30 batches of reaction, the deteriorated pack has to be taken off from the inlet side of reactor column and a new pack is added on the outlet side. Therefore, the reaction is carried out by batchwise recycling the penicillin G solution through the reaction column, at the temperature of 30-36°C. The pH is maintained at constant level through automatic addition of 4N NaOH solution. After batchwise reaction, the reaction mixture is transferred to a crystallizer vessel and methanol (about half the volume of the total reaction mixture) is added. 6-APA is precipitated from the solution by adjusting the pH with 6 N hydrochloric acid. The precipitate is filtered, washed with methanol and dried in vacuo. These steps yield 6-APA which is more than 98% pure, and a total yield of 86% from theoretical yields.

136. In the Toyo Jozo process, the conditions are as follows. One batch of reaction gives 125 kg of 6-APA, and it takes 3-3.5 hr recycling time. Two batches of reaction are carried out per day. The main equipment of the plant includes: jacketed 5,000 l vessel as reservoir; 200 l of 4 N NaOH tank connected to automatic valve for pH regulation; 198 l reactor column which is composed of 6 layers of the enzyme-bed; recycling pump with a capacity of 100,000 l/hr, 37 KW; two 7,000 l of jacketed vessels as crystallizers; vacuum evaporator; filter press.

137. Snam Progetti performs the hydrolytic reaction batchwise, recycling through a column, packed with the fibre-entrapped penicillin amidase (strings made of these fibres are strung parallel to the axis of a tube), a water solution of penicillin or cephalosporin (6-12%, w/v) at a temperature of 37°C and a pH value of about 8.0 is kept constant by continuous automatic addition of an alkaline solution. Once a hydrolysis degree of 98% is reached, the reaction mixture is conveyed to the 6-APA or 7-ADCA recovery tank, and a fresh antibiotic solution is fed into the reactor. The product is precipitated by adjusting the pH of the reaction mixture to its isoelectric point (pH 4.2) in the presence of an organic solvent miscible with water; the precipitate is filtered, washed, and finally dried under vacuum. A purity level of the resulting 6-APA is usually higher than 96-98%, and the overall yield is between 85 and 90% (12). Snamprogetty indicated, that the company's plant operates for 15 hr over 24 hr and carries out 4 batches lasting 3 hr each. Half an hour is needed, after each batch, to discharge the hydrolyzed penicillin solution and start a new batch (60).

138. Snam Progetti indicated, that the life of penicillin amidase immobilized in fibres under operating conditions is strictly dependent on the purity of cephalosporin G when 7-ADCA is the product of the enzymatic conversion. Provided that the purity level of cephalosporin is higher than 95%, it was estimated than one kg of enzyme fibres (dry basis) produce about 150 kg of 7-ADCA. The overall yield of 7-ADCA is of 85-90% (63).

139. The equipment for the 12 ton/year Snam Progetti's plant includes: 40 l tank for soda solution; two stainless steel stirred jacketed 300 l vessels; one enzymic stainless steel 100 l reactor containing penicillin amidase fibres; one recycling centrifugal pump with a capacity of 15,000 l/hr; one 100 l tank for HCl solution; one NaOH solution pump with a capacity of 12 l/hr; one stirred stainless 2,000 l vessel; one centrifugal separator with basket capacity of 70 kg; one vacuum dryer, maximum temperature of 40°C (60).

140. In the Tanabe Seiyaku Co. process, a solution of 0.05 M penicillin G in 0.01 M borate-phosphate buffer (pH 8.5) was passed through the column packed with **E.coli** cells immobilized in polyacrylamide gel at 30°C. From the column effluent 6-APA was obtained in about 80% yield based on the used penicillin G (53). The half-life of the immobilized catalyst at these operating conditions was equal to 42 days at 30°C or to 17 days at 40°C.

141. In the Chinese industrial process (see paragraph 130) after 285 cycles of 6-APA production over 7.5 months, the hydrolytic rate was maintained unchanged (59). According to another source (64), the immobilized cells column for 7-ADCA production was used 23 times during 30 days without a decrease of activity; a similar column was operated on an industrial scale 72 times over a 3-month period.

142. An industrial plant for 6-APA production using immobilized disrupted bacterial cells containing penicillin amidase has been using since 1979 in Slovenska Lupca (Czechoslovakia). The half-life of the cross-linked catalyst (see paragraph 129) is a minimum of 500 cycles (2 hr each) in a batch reactor at 37°C, pH 7.8.

143. According to a Genin company representative (paragraph 133), genetically engineering E.coli cells entrapped in carrageenan yield 90% or higher, 6-APA with the 6-APA concentration up to 70 g/l. In their process, penicillin G is pumped down the column of a packed bed reactor. In the scaled-up pilot plant of Genin the enzymatic reaction has been carried out with 3,000 litres of working volume, and the specific activity of the cells is comparable to that obtained in an 8-litre reactor (61).

144. Hindustan Antibiotics Ltd. described their pilot plant which produces 6-APA using penicillin amidase covalently bound to modified cellulose (56). The enzymatic reaction proceeds in a continuous stirred 100 litre reactor, where penicillin G solution is added to the enzyme suspension (50 g/l) in a ratio of 2:1 (v/v), and the final volume of the reaction mixture is then adjusted to 10:3 with water. The reaction was kept stirred at 40°C during the course of reaction and pH was maintained between 7.5 to 7.8 by addition of alkali. The quantity of bound enzyme used for conversion of 4.6 kg of penicillin G was 3.6 kg (containing 371 g of protein).

145. A fully automated computer controlled batch reactor for 6-APA production is described (65), where penicillin amidase immobilized on Eupergit C, made by Rohm Pharma, acts as a catalyst (see para. 134). The reactor performed more than 400 reaction cycles using the

same catalyst, and the productivity of the enzyme was given as a minimum of 20 tons of 6-APA (isolated product of 99% purity)per 100 kg of catalyst. One cycle runs for 80-120 min reaction time with 8% of penicillin G (potassium salt) and 8% of wet catalyst, with a conversion rate of 98-99% and a product yield of 50% (ref.weight of penicillin G - potassium). With the average reaction time of 100 min, the filling time of 20 min and the emptying time of 15-45 min (see par. 134), a complete cycle takes about 3 hrs. Thus, in a 24-hrs operation, 8 batch cycles can be carried out (62).

146. An industrial process in the USSR operates with immobilized penicillin amidase (par. 129) which performs about 700 reaction cycles, up from 300 at the end of the 1970's and from 100 at the beginning of the 1970's. The total yield of 6-APA is equal to 86-88% (66).

D. Economic estimates

147. There are economic estimates available from Snam Progetti (Italy) for the production of 6-APA integrated with the manufacture of penicillin G itself (12). In this case the overall yield in 6-APA bypassing the crystallization of penicillin G, was higher. Shown below is a comparison between the conventional process using penicillin amidase immobilized in fibres as well as the integrated one with respect to the chemical method. For the integrated process a lower cost was calculated because the cost of utilities, chemicals, and labour encountered in the crystallization of penicillin G disappeared. The calculations were based on a plant capacity of 40 ton/year, penicillin G price of $ 14.35/BU.

6-APA manufacturing cost (U.S. dollars per kg)

	Chemical Process	Conventional Enzymatic Process	Integrated Enzymatic Process
Penicillin G	43.72	46.01	39.65
Chemicals	7.49	0.78	1.35
Fibre entrapped amidase	-	2.67	2.67
Utilities	4.64	0.91	0.91
Labour	3.12	3.95	3.95
Operation supplies	0.29	0.36	0.36
Depreciation	6.16	4.99	3.85
Other overheads	2.99	2.82	2.54
	68.41	62.49	55.28

Source: ref. 12

148. Economic estimates showed that the application of immobilized penicillin amidase on the industrial level in the USSR has accounted for 90 million roubles (US $ 130 million) saved for the period of 1978-1985 (66).

E. Scale of the processes

149. 6-APA as an important intermediate in the synthesis of the semisynthetic penicillin is currently produced by Astra Company in Sweden, by the Bayer Company in West Germany, by Beecham in England, by Minmedbioprom in the U.S.S.R., by Novo in Denmark, by Spofa in Czechoslovakia, by Squibb Company in the U.S.A., by Toyo Jozo Company in Japan, by an industrial plant in China, and possibly, some others.

150. According to published data, Toyo Jozo in Japan produces 5 tons of 6-APA per month; Spofa, operating through "Biotika" plant in Slovenska Lupca, produces 30 tons of 6-APA per year. Pilot plants have been operated for some time by Snam Progetti in Italy, Tanabe Seiyaku Co. in Japan, Hindustan Antibiotics Ltd. in India, Genin in Mexico. All the 6-APA produced in the Soviet Union and Japan is manufactured by means of immobilized penicillin amidase. According to (29), approximately 4500 ton/year of 6-APA is produced worldwide with an estimated enzyme usage of 4-5 ton/year.

VII. USE OF IMMOBILIZED LACTASE IN DAIRY PROCESSING

151. The use of lactase (ß-galactosidase) in the dairy industry offers one of the most promising commercial exploitations of immobilized enzymes. Lactase hydrolyzes lactose, the principal carbohydrate of milk, a sugar with poor solubility properties and a relatively low degree of sweetness, to galactose. The relative sweetness of lactose is 20-40 (in different conditions) whereas that of glucose and common sugar (sucrose) is equal to 70-75 and 100, respectively. The hydrolysis of lactose offers some advantages to a dairy product. First, it improves the digestibility of lactose; it is well known that some infants, adults, and some individuals of all ages of certain ethnic groups are unable to digest lactose probably because of the lack of lactase in the small intestine mucosa (12). In that case the administration of milk products causes abdominal pain, cramps, diarrhea, and general intestinal upsets. This problem can be circumvented if lactose in the product is hydrolyzed by lactase to the readily utilizable sugars, glucose and galactose. Then, low solubility of lactose can result in its crystallization in dairy food causing defects such as sandy or gritty texture, deposit formation and protein destabilization. Products which suffer most from such crystallization are condensed milk, sweetened condensed milk, condensed and dried wheys, ice cream and frozen milks. Using lactase in such products could reduce lactose concentration to a point where it no longer presents a problem (67). Another advantage is the possibility of preparing or improving new food and dairy products with higher solubility characteristics and a higher degree of sweetness.

152. Moreover, lactose is the major by-product of cheese manufacturing that causes many pollution problems, and its utilization becomes more and more interesting because of the need for environmental protection. Modern dairy products sometimes produce over 1 million liters of whey daily, and traditional means of whey disposal are no longer acceptable. Because whey and ultrafiltration by-products from the manufacture of cheese or whey protein have very high biochemical oxygen demand (of about 50 gm O_2/l), they are great environmental hazards and cannot be discharged without expensive treatment (68). Whey or ultrafiltration by-products can be upgraded by means of the enzymatic hydrolysis of lactose by lactase.

153. By-production of whey in the U.S.A. alone exceeds 15 million tons a year (69), of which only one-half is further processed. France produces 7 million ton/year of whey, of which protein constitutes 45-50 thousand tons, and lactose - 330-350 thousand tons (70). The British Food Association reported that 24% of 103 surveyed food companies dealt with the utilization of dairy wastes (71). The economic potential of hydrolyzed lactose syrup has been calculated at approximately $ 35 million per year (69). This estimate is based on the relative sweetness of the sugars, a 70% conversion of lactose to monosaccharides and a value of $ 0.22/kg for sucrose. Clearly as the price of sucrose increases, the economic potential of hydrolyzed lactose increases.

A. A background

154. Cheese whey, the major by-product of the dairy industry, contains about 6.5% solids of which 70% is lactose. Thus, lactose is the main solid ingredient of whey, constituting 4-5% of dry mass. Lactose can be hydrolyzed by using ion exchange resins, strong mineral acids, and enzymes. Ion exchange resins and strong acids tend to destroy the whey protein through irreversible denaturation. The use of hydrolyzing enzymes lowers the lactose content without adversely affecting the proteins and other components in whey. Through immobilization, enzymes can be repeatedly used for lower operating and separation costs. Milk treated by the enzymatic methods retains its original nutritional value because glucose and galactose, the products of lactose hydrolysis, are not removed.

155. Lactase hydrolyzes the lactose in whey or milk to equal quantities of glucose and galactose. Compared with the sweetness of sucrose (taken as basis with a relative value 100) the glucose-galactose syrup has a value of about 70 which is similar to that of glucose and is considerably sweeter than lactose itself (see paragraphs 19 and 151). Hydrolyzed lactose solutions can be evaporated to concentrated syrups, since glucose and galactose are both very soluble, unlike that of lactose. The syrups with 70-80% sugar content are fluid and have a good shelf life.

156. Only 25% of the lactose needs be hydrolyzed to prevent crystallization during prolonged storage, because small amounts of glucose and galactose inhibit the formation of lactose crystals. This prevents ice cream and frozen deserts from acquiring a sandy texture due to crystallized sugar. The need for stabilizers is thereby eliminated.

B. Commercial preparations of immobilized lactase

157. Lactases occur rather widely in nature and have been isolated from animals, plants, and microorganisms. Enzymes of plant and animal origin are of little commercial value but several microbial lactases are of technological interest. The major enzymes of commercial interest are those from yeasts **Kluyveromyces** or **Saccharomyces lactis, Kluyveromyces** or **Saccharomyces fragilis** and fungi **Aspergillus niger** and **A.oryzae**.

Commercial preparations of lactase

Source	Company
Aspergillus niger	Baxter Laboratories, Chicago, IL, USA Dairyland Food Labs,Waukesha, WI, USA Kyowa Hakko Kogyo Co., Japan Societe Rapidase, Seclin, France Wallerstain Co.,Morton Grove,IL, USA GB Fermentation Industries, Inc., IL,USA
Kluyveromyces or Saccharomyces lactis	Gist-Brocades, Delft, Holland ("Maxilact") Nutritional Biochemicals Co. Ltd., Cleveland, OH Tokyo Tanabe Co., Ltd., Tokyo, Japan
Kluyveromyces or Saccharomyces fragilis	Kyowa Hakko Kogyo Co., Japan Sigma Chemicals Co., St Louis,MO, USA Novo A/S, Denmark ("Lactozym")
Escherichia coli	C.F.Boehringer GmbH,Mannheim, FRG Worthington Biochemical Corp., Freehold, NJ, USA
Yeast preparations	British Drug House Ltd, London, England DEBI, Cassina de Pecchi, Milan, Italy Sturge Enzymes Ltd., England ("Hydrolact") Miles Laboratories, USA ("Godo")
Fungal preparations	Miles Laboratories, USA ("Takamine")

Source: ref. 72

158. Lactases are rather expensive. Typical prices for the partially purified enzymes are: **K.lactis** $ 1.02 per mkatal, **A.niger** $ 7.56 per mkatal, **E.coli** $ 660 per mkatal (assuming that 1 mkatal will hydrolyse 1 mmole or 0.23 g of lactose per second under optimum conditions). These prices make it essential that the enzymes be immobilized if they are to be used in large-scale manufacturing processes (69).

159. The fungal lactases (from **Aspergillus**) with acid pH optima (2.5-4.5) are especially suitable for hydrolysis of lactose in acid whey, but they are more sensitive then others to product inhibition (namely inhibition by galactose). The yeast enzymes (from **Kluyveromyces** or **Saccharomyces** sp.) have neutral pH optima (6-7) making them suitable for the hydrolysis of lactose in milk or sweet whey, and they are also less inhibited by galactose. However, they are less thermostable than the fungal enzymes. Besides,the use of the yeast lactases is limited because they need activating ions to reach their full activity level. Of the bacterial lactases only the one derived from **E.coli** (pH optima 6.5-7.5) is available commercially, in both crude and pure form. It is not used, however, in food processing because of its cost and the fact that it causes toxicity problems when crude extracts of coliforms are used.

160 Not all lactase sources are acceptable or have GRAS (generally recognized as safe) status when the enzyme is going to be used in food systems. Lactase preparations from **A.niger, A.oryzae** and from **Saccharomyces** sp. (lactis or fragilis) are considered safe because these sources already have a history of safe use and have been subjected to numerous tests (72). Currently GRAS status is valid for **A.niger, A.oryzae, K.fragilis** and **K.lactis** (72,73) for use in the production of lactase-treated milk and lactose reduced milk.

161 Because of the rather high price of lactases as compared to the low value of the waste product whey, the direct addition of lactase to the substrate is economically prohibitive. This problem can be overcome by immobilization of the enzyme. In this way a variety of techniques and support carriers have been used. However, from a list of lactase immobilized commercial preparations shown below a preference for some specific methods and immobilizing agents is quite obvious: adsorption and cross linking with glutaraldehyde, and covalent coupling with a solid carrier.

Commercially available immobilized lactase preparations

Company	Immobilization procedure
Snampogetti	**K.lactis** lactase entrapped in cellulose triacetate fibres by means of fibre wet spinning
Corning Glass Works	**A.niger** lactase covalently bound to silica beads. Parcile size 0.4-0.8 mm, density 0.6 g/ml.
Connecticul/Lehigh Universities	**A.niger** lactase adsorbed on porous and cross-linkedwith glutaraldehyde. 50% binding efficiency.
Diamond Shamrock	Enzyme adsorbed on ion-exchange resin
University of Lund	Enzyme entrapped in polyacrylamide gel
Valio Laboratory	**A.niger** lactase adsorbed/crosslinked to phenol for aldehyde resin Diolite ES-762, particle size 0.25-0.35 mm. Enzyme loading 25 mg/g of resin.
Sturge	**A.niger** lactase ("Hydrolact") covalently coupled with silanized Mn-Zn ferrite nonporous particles
Gist Brocades	**Sacc.lactis** lactase ("Maxilact") entrapped in cellulose triacetate fibres by wet spinning method, 30 mg/g dry fibre.
Wallerstein	a) Enzyme entrapped in polyvinyl alcohol gel. b) Enzyme entrapped in hollow fibre membrane, 2-59 g/m^2
Rohm GmbH	**A.oryzae** lactase covalently bound to macroporous 0.1-0.3 mm beads made of plexiglas-like material; pores 0.1-0.3 microns
Sumitomo	**A.oryzae** lactase covalently bound to a macroporous amphoteric ion-exchange resin
Amerace Corp.	**A.oryzae** lactase covalently bound to a microporous PVC-silica sheet

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Source: ref. 72

162. Glutaraldehyde as a bifunctional cross-linking agent is usually accepted in the food industry and moreover presents disinfecting properties (72). Phenolformaldehyde resin is often the carrier for this method. It is used in scaled-up systems in the form of fluidized bed reactor. Another common carrier related to covalent coupling method is porous silica in the form of beads. Coupling of the activated beads to the enzyme is commonly obtained through glutaraldehyde. A similar technique is used with other inorganic carriers like porous alumina and ferrites.

163. Rohm GmbH (Darmstadt, FRG) has used for immobilization of the **A.oryzae** lactase a macroporous, bead-shaped carrier with a porosity of 3-4 mg/g, where the enzyme was bound covalently by oxirane groups. Because of their rigid structure the 0.1-0.3 mm diameter beads are pressure stable and show "good flow properties" (37). The immobilized lactase has the brand name of Plexazym LA. The authors claimed (74) that the immobilization method produces a much higher immobilization yield than standard immobilization techniques (adsorption, gel entrapment, microencapsulation). The beads are hard and resistant to compression, similar to an ion-exchange resin. According to Rohm GmbH, 1 kg of the immobilized lactase can hydrolyze 80% of 960 litres of whole whey within 24 hr (74).

164. The immobilized **A.oryzae** lactase was developed recently at the Technisch-Chemisches Laboratorium, RTH Zentrum in Zurich, Switzerland, where the enzyme was adsorbed on Duolite S-761, ion exchange resin, and cross-linked by glutaraldehyde. The price per unit activity of immobilized enzyme, including the carrier price and enzyme deactivation, was found to be optimal at a level of 20 mg enzyme/g Duolite (68). The half-life time for the immobilized enzyme was 31 days at the operating conditions (40°C, pilot plant, deionized whey).

165. Snamprogetti has used K. Lactis lactase, namely a purified commercial preparation Maxilact (from Gist Brocades, Delft, Holland). To prepare the fibre-entrapped lactase, the enzyme powder was suspended in a buffer (pH 7.2, 1 mM EDTA and 2 mM $MgSO_4$) and allowed to stand for 24 hr at 4°C with gentle stirring. Insoluble material was removed by centrifugation and a 30% (w/w) solution of clarified extract in glycerol was prepared. The solution was entrapped in cellulose triacetate according to the standard procedure by Snamprogetti (see para. 25).

166. Lehigh Valley Dairy (Allentown, Pennsylvana) in their pilot plant has used the immobilized lactase-on-aluminia catalyst (75). The catalyst was A.niger lactase immobilized on 150 micron diameter porous alumina particles of pore diameter of about 4,000 A, and surface area of about 4 m^2/g and showed "good fluidization characteristics and long term mechanical, biological, and chemical stability during operation" (75). Less than 0.5% of the catalyst was lost by attrition and elutriation from a fluidized reactor over a 2-week period.

167. Corning Glass Works (Corning, N.Y.) patented recently lactase immobilized on microporous ceramic beads and showing "improved immobilized enzyme stability ... by a factor of about 300" (U.S. Pat. 4,409,247). Earlier preparations of the immobilized enzyme were comprised of **A.niger** lactase chemically bound to silane functional groups on the treated porous glass beads surface. Such immobilized systems having upto 50 mg if enzyme bound to one gram of porous glass, generally retained 30-80% of the activity of the free enzyme. Each gram of lactase-coated glass could hydrolyze about 6 grams of lactose per hour at 35°C (76).

168. Sumitomo Chemical and Shin Nippon Chemical Industries have developed "high-purity immobilized lactase for use in hydrolysis of milk sugar" (77).

C. Technological characteristics of the processes

1. Production of low lactose milk

169. The enzymatic hydrolysis of lactose can be achieved either by soluble enzymes, usually in batch fermentation process, or by immobilized enzymes. Although the subject of this review is immobilized enzyme systems, the following table represents some data on soluble lactase systems which have been used to diminish lactose content in milk. High residence times are usually necessary for such systems (several hours to several weeks) as compared with that for immobilized enzyme (usually several minutes).

Soluble lactase systems

Source of enzyme	Substrate	Enzyme load	Temperature (°C)	Conversion degree (%)	Residence time (hr)
"Lactozym"	Milk	0.1-0.2%	40-45	70-80	4
S.fragilis	Skim milk	64/g lactose	35	75	6
"Lactozym"	Skim milk	5,000 U/l	40-45	90	4
K. lactis	Milk	0.54 kg/m3	4	62	18
"Lactozym"	UHT milk	0.03 ml/l	20	70-8-	1 month

Source: ref. 72

170. Currently, two companies use large-scale processing of milk by means of soluble lactase (72). The Valio Laboratory's system uses 0.4 kg/m^3 of **K.lactis** lactase ("Maxilact" 40,000) to treat 40,000 of UHT milk per day. Tetra Pak company uses a technology, in which milk produced and bottled aseptically is hydrolyzed by "Lactozym" during storage at room temperature. 70-80% hydrolysis occurs in one month, requiring only 0.01-0.03 ml of enzyme per litre of milk.

171. The only industrial process for the treatment of milk with the immobilized enzyme system has been operated by Centrale del Latte of Milan, Italy, since 1977, utilizing the Snamprogetti technology. The immobilized enzyme is lactase from yeast (a purified commercial preparation Maxilact, from Gist-Brocades, Delft, Holland) entrapped in triacetate cellulose fibres (see par. 161, 165), and the reaction is performed batchwise at low temperature. The processed milk, after having reached the desired degree of hydrolysis of lactose, is separated from the enzyme fibres, sterilized, and finally sent for packing and distribution.

172. The flow chart for the Snamprogetti's pilot plant which preceded the industrial plant is shown in Figure 10. The pilot plant has been used for carrying out the hydrolysis of milk lactose since 1974. It comprised essentially a 300 l stainless steel receiving tank, a continuous sterilizer (Cherry Burrel, Pilot, Cherry Burrel Co., Cedar Rapids, Lowa, USA), a 20 l jacketed glass column (internal diameter,

15cm; height, 56 cm) containing the entrapped lactase (4 kg of wet fibres), a peristaltic pump, and a 500 l aseptic jacketed tank. The glass column and the aseptic tank were thermostated at 4-7°C. The lactase fibres were placed parallel to the column axis and fixed at their two extremities. Raw skimmed milk was allowed to pass through the sterilizer (sterilization temperature 142°C, for 3 sec holding), rapidly cooled to 4 to 7°C, and pumped to the aseptic tank through the column at a flow rate of 7 l/min. The recirculation was continued until the desired lactose conversion was obtained. As has been shown, no contamination of treated milk occured, e.g. because of the leakage of enzyme from fibers (46). In fact, the loss of activity of a 0.5 kg sample of enzyme fibre that processed about 10,000 litres of milk in 50 batches was less than 10 % (12).

173. Because microbial contamination in this process may cause serious problems, care has been taken in assembling the pilot plant with a unit where all the piping and related plant equipment, except the enzyme reactor, were sterilized by steam. This kept the bacterial count in the product within the limits for pasteurized milk (in Italy less than 30,000 per ml). In fact, the total bacterial count at 32°C in the product was always less than 10,000 per milliliter (12,46). As regards the quality of the product obtained, tests performed have demonstrated that "the organoleptic characteristics remain unaltered except for a very slight increase in sweetness" (46). As regards the shelf-life of the product, it "can be stored at least 3-4 months if kept in refrigerator at 4°C" (46).

174. Rohm GmbH has developed a pilot plant for processing whole milk (3.5% fat, 4.6% lactose), using Plexazym LA (see paragraph 61). Because of the optimal heat stability of the immobilized enzyme at neutral pH, the packed bed reactor has been operated at 55°C. At a space velocity of 20 hr^{-1} a productivity of 13 tons of milk/kg Plexazyme was reached in 30 days. The packed bed did not get plugged by the droplets of the fatty emulsion or by the casein micelles of milk because of the morphological properties of the support (37).

2. Processing of whey

175. Before 1980 processes using immobilized lactase had been operated mainly on pilot plant scale. Probably the first pilot plant was that of Valio Cooperative Dairies Association in Finland, who began in 1976 operating their 5 ton/day continuous column bioreactor for cheese whey lactose hydrolysis by phenolformaldehyde resin bound **Aspergillus niger** lactase (78). The technology had been developed by Valio in co-operation with the Technical Research Centre of Finland. Since 1978 whey has been processed on an industrial scale both to improve the digestibility of pig feed and to obtain hydrolyzed lactose products for various food applications (78).

176. One of the first pilot plants was developed jointly by Lehigh University (Bethlehem, Pennsylvania), University of Connecticut (Storrs, Connecticut), and Lehigh Valley Dairy (Allentown, Pennsylvania). The pilot plant hydrolysis unit comprised two 2 m

fluidized-bed of 7.6 cm I.D., each containing about 4 kg of catalyst (**A. niger** lactase adsorbed on a porous alumina and cross-linked with glutaraldehyde) and having a maximum daily flow-through capacity of about 160 l of raw (unfiltered) acidic cheese whey. Both columns were equipped with conical inlet sections above quick-acting ball valves which could start or stop liquid flow without loss of catalyst from the bottom of a column. It has been found in practice that such conical inlets could provide good liquid distribution and minimize channelling during operation. Besides, ultrafiltration and ion exchange capabilities have been included in the pilot plant in a scheme which permitted any sequence of one or more of the three operations. The flow diagram is shown in Figure 11 (75).

177. With reference to Figure 11, the whey was first heated to the desired hydrolysis temperature (45 to 60°C) in a stainless steel shell and tube heat exchanger (American Standard SSCP-D3014) from which it was pumped through the fluidized-bed immobilized enzyme hydrolyzers, with a flow rate of 0.5 l/min, pH 4.9. Hydrolyzed whey then flowed through a particle trap which removed any catalyst which might elutriate. Subsequent flow was to the ultrafiltration and/or ion exchange units if post hydrolysis treatment was desired. Very little of the catalyst was elutriated; a liquid fluidized bed was characterized by very gentle agitation of the catalyst particles and a very sharp liquid catalyst interface at the top of the bed. If prehydrolysis treatment was desired, raw whey could be ultrafiltered and/or demineralized. Ultrafiltration was achieved by a Romicon (1115-45-XM50) filter, which had a molecular weight cutoff of 50,000 and an average flux of about 600 l/m^2/day and permitted recovery of valuable whey protein. Ion exchange was used for demineralization. The pilot plant demineralization consisted of an anion exchange column containing Duolite ES-340 resin and a cation column containing Duolite C-20 resin (75).

178. When raw, unfiltered whey was fed directly to the hydrolyzer, some difficulty was experienced in maintaining constant temperature during these experiments. Then, there was a slow but discernable decrease in conversion which could not be completely explained by the temperature variations experienced. The sanitization with Iosan involved washing the bed in the expanded state for approximately 1 hr; an increase in activity (and hence enhanced conversion) was observed directly after the sanitization, which, however, was lost soon after the feed of raw whey was started again, and the slow decrease in conversion continued. Based on the pilot plant experiments a plant able to process 50 tons of whey/day was projected (75), taking into account the following aspects of the process:

-lactose conversion 70 %
-catalyst half-life 60 days
-total catalyst required 710 kg
-reactor diameter 51 cm
-reactor height 6,9 m

The economic estimates of the plant are given in paragraphs 198-200.

179. Snamprogetti has used its pilot plant with yeast lactase entrapped in cellulose triacetate fibres (see par. 165) for the hydrolysis of lactose in sweet whey (12). Proteins were separated from the whey by ultrafiltration; the permeate, containing the mineral salts and lactose, was continuously fed to the enzyme reactor at 60°C, pH 4.5. the effluent from the reactor was demineralized by ion-exchange treatment and concentrated to 80% total solid. The following parameters for the 100 l of whey/day reactor have been estimated:

-lactose conversion	95%
-catalyst average life	90 days
-total catalyst required	1 kg (dry mass)
-reactor diameter	8 cm
-reactor height	1 m
-temperature	60°C
-flow rate	16 l/hr

180. Snamprogetti also developed a 1000 l reactor for the hydrolysis of lactose in raw, untreated sweet whey. The stainless steel reactor of 100 cm in height and 120 cm in diameter, containing 5 kg (dry weight) the entrapped yeast lactase, operated at 25°C, pH 6.5-7.0 for 3.5 hr to reach 95% of the conversion of lactose. Average life for the catalyst was 90 days.

181. According to Corning Glass Works' data (79), two important variables were found to affect the observed half-life of immobilized lactase in operating conditions: feed composition and temperature. From the table shown below it can be seen that the higher the feed purity, the longer the half-life. The normal salt content of the whey feed was detrimental to enzyme half-life although it affected activity insignificantly. Removal of 90 to 95% of the salt in deproteinized acid whey resulted in dramatically improved enzyme stability.

Effect of feed composition on half-life of A.niger lactase immobilized on porous silica by the aqueous silane glutaraldehyde method

Feed	Average half-life at 50°C (days)
Whole acid whey	8
Deproteinized acid whey	10
5% lactose/0.5% NaCl	13
De-ionized, de-proteinized acid whey	60
5% lactose	100

Source:ref. 79

In fact, a half-life of 62 days at 50°C was determined experimentally for the hydrolysis of lactose in deproteinized, de-ashed acid whey in the pilot plant's reactor (Fig. 12) vertical cylindrical column of 10 cm diameter and 70 cm enzyme bed height (79). The column operated down flow. Figure 13 indicates the general principle of the operation of the modified hydrolysis unit (80). The process is run at constant throughput, constant conversion, with the temperature slightly raised to compensate for the thermal de-activation of the composite. Cleaning is achieved by backflushing the bed with dilute acetic acid. The estimated life of such a system is around two years under laboratory conditions (80).

182. Rohm GmbH has used a pilot plant with packed bed reactor containing Plexazym LA (see paragraph 161) in order to hydrolyze lactose in filtered acid whey at pH 4.5, with a space velocity of 50 hr^{-1} (37). The rate of hydrolysis decreased from an initial value of 100% to about 90% over 60 days of operation. After 100 days of operation, the degree of hydrolysis was still 80%. The productivity of 70 tons of whey/kg Plexazym LA was reached in 60 days. The pilot reactor by Rohm GmbH contained 1 kg of the immobilized lactase. A day's run consisted of 20 hr of hydrolysis (960 l of whey was typically hydrolyzed) and 4 hr of cleaning with a 0.1% solution of a quarternary ammonium salt (74).

183. A fully automated pilot plant "Lactohyd" for the enzymatic hydrolysis of lactose in sweet whey is under operation at the Technisch-Chemisches Laboratorium, EHT Zentrum, Zurich (Switzerland), and its simplified flow diagram is shown in Figure 14. The plant contained fixed bed reactors with a total capacity of 800-1000 l whey/day (68). **A.niger** (then **A.oryzae**) lactase immobilized on the ion exchange resin, Duolite S-761 by adsorption and cross-linking with glutaraldehyde (see paragraph 164) is used as the enzyme catalyst. "Quickfit" glass was used for easy plant construction and visual inspection under operating conditions. Four fixed bed reactors were installed so that every combination of their connections was possible. Actually three of them were used for hydrolysis with **A.niger** lactase, with the fourth on stand-by, waiting to be exchanged with any reactor in need of **in-situ** regeneration. This reactor in turn was put on stand-by after its regeneration. In fact, only one reactor - instead of three used with enzyme from **A.niger** - was required to achieve 90-95% lactose conversion with **A.oryzae** lactase, which was more active (68).

184. The pilot plant "Lactohyd" has been operated in a mode with catalyst regeneration and re-immobilization performed **in situ**. During the plant's operation, microbial contamination "was of no problem" (68). The basic features of the pilot plant are listed below:

Overall performance	800-1000 l whey per day
Production cycle	20 or 40 hrs
Sanitation cycle	4 hrs
Automation	Complete for both cycles
Sampling	Automated
Half-life time for the immbilized enzyme catalyst	31 days (at 40°C), deionized whey
Type, enzyme reactors	Fixed bed, particles 0.4-0.8 mm
Catalyst volume	3 l/reactor
Mode of operation	Asynchronous reactors, temperature increase with time, constant flow rate
Sanitation	Counter-current flushing
Regeneration	**In situ**

Ultrafiltration	30-40 l permeate/hr, 1.6 m^2 membrane area
Evaporation (preconcentration)	5-10 wt-% lactose
Evaporation (syrup)	60-70% dry mass
Whey protein concentrate	75-120 l/day
Glucose/galactose syrup	50-70 l/day 75-95% hydrolysis 60-70 wt-% dry mass

Source: ref. 68

185. In 1978, at the Milk Marketing Board England and Wales Technical Division (Crudgington, Telford Salop, U.K.) facility, a semi-industrial plant for the enzymatic hydrolysis of lactose in cheddar sweet whey was put into operation (80). The production of the plant has been used to develop the application of hydrolyzed lactose syrups. **A.niger** lactase was immobilized according to Corning Glass Works' method, that is, by means of covalent binding to a controlled-pore silica carrier using the silane-glutaraldehyde technique (see paragraph 161). The plant has been operated on a continuous basis five days a week and processed about 30,000 liters of the whey a week resulting in about 1.7 tons of syrup (1200 kg of solids).

186. The whey pathway in the plant was as follows. Raw whey was pasteurized and sent to an ultrafiltration plant. The permeate fraction was then de-ashed by passage over an ion-exchange demineralization unit, and held in a standard dairy tank for storage. The hydrolysis plant operated at 360 l/hr and achieved 80% hydrolysis. The hydrolysis plant was entirely automatic and operated 16 to 20 hours per day. It was self-protected by a series of alarms and automatic shut-down procedures. When no more feed was available at the end of a run, the plant used to shut itself and was ready for the cleaning cycle. While the system was cleaned by standard dairy cleaners, the lactase bed was fluidized every day for half an hour with dilute acetic acid. The temperature varied from 32 to 39°C during half-a-year operation, and the enzymatic system showed very good stability (80).

187. A similar pilot plant has been concurrently running by Union Laitierre Normande in Conde/Vire, France, with the main goal of examining other feeds and operating conditions (80). The pilot facility installed there had a capacity of 500 l/hr at 80% conversion. The plant was run four days a week, 6 to 8 hours per day. Daily cleaning took 2 to 3 hours. During these runs different kinds of whey feed have been used (casein and whey, sweet whey, etc.), and various models of whey pretreatment were studied, electrodialysis partial demineralization in particular (80).

188. The Nutrisearch Co., a joint venture of the Kroger Company and Corning Glass Works begun in 1982 (see paragraph 204), has built a new $ 15 million plant in Winchester (Kentucky, USA) which uses the immobilized enzyme technology of Corning and continuous fermentation technology of Kroger (81). The plant operates in three

stages: these are, protein removal from cottage cheese whey in an ultrafiltration system, hydrolysis of lactose in the perfusate with the immobilized lactase, and fermentation by baker's yeast of the simpler sugars formed. In a 20-hour operating day the Nutrisearch plant converts 400 tons of raw cottage cheese whey into protein concentrate and baker's yeast. The plant is located next to Kroger' Winchester Farms Dairy, from which raw whey is pumped through an underground pipe and stored in 120 m^3 raw whey tanks. Whey is also trucked in from other Kroger dairies in the midwest. Reverse osmosis systems have been installed at these dairies to remove one-half to two-thirds of the water from the whey to reduce shipping costs. When whey is received, it is centrifuged and sterilized in a typical dairy type high temperature-short time plate pasteurizer. The pasteurized whey is stored at 55°C in 160 m^3 tanks (81).

189. Before the enzymatic hydrolysis step, the protein is removed from the whey in a four-stage ultrafiltration system. The permeate, containing 95% of the liquid and lactose, is pumped to three 200 m^3 tanks for in-process storage. From these, the stream, which contains about 5% lactose by weight, is split, and pumped to the two hydrolysis columns. The retentate after the ultrafiltration, which contains most of the whey protein, leaves the unit at approximately 20% total solids. About two-thirds of these solids are protein, 30% lactose and the reminder is minerals (81).

190. The hydrolysis columns are 1 m diameter, 5 m high stainless steel pressure vessels filled with the immobilized enzyme (30/45 mesh particles), and operate in the downflow mode. Temperature in the columns is controlled at about 20°C. Product (glucose-galactose syrup) from the hydrolysis columns is pumped into 80 m^3 storage tanks and then directed to the fermentation stage (81).

191. Summed up below are data available on technological applications of immobilized lactase.

Technological applications of immobilized lactase

Company	Substrate	Catalyst	Applications and Scale
Snam Progetti; Centrale del Latte (Italy)	Skimmed milk	Yeast enzyme entrapped in triacetate cellulose fibres	Industrial, 10 ton/day
Snam Progetti	Sweet whey permeate	same	Pilot, 100 l/day, conversion 95%
	Raw sweet whey	same	Pilot, 1000 l/day conversion 95% within 3.5 hr

Milk Marketing Board (U.K.), Corning Glass Works	Cheddar sweet whey permeate	same	Semi-industrial, 30 tons of whey/ week, conversion 80%
Union Laitierre Normande (France), Corning Glass Works	Different kinds of whey	same	Pilot, 12-16 ton/ week, conversion 80%
Technisch-Chemisches Laboratories, EHT Zentrum (Switzerland)	Sweet whey	Fungal enzyme adsorbed on ion exchenge resin and cross-linked	Pilot, 800-1000 l/day, conversion 75-95%
Nutrisearch Co. (USA)	Cottage cheese permeate	Fungal enzyme covalently bound to silica	Industrial, 400 ton/day
Valio Laboratory, Keymenlaakso Dairy (Finland)	Whey permeate, whole milk	Fungal enzyme adsorbed to phenol formaldehyde resin Duolite ES-762	Industrial, 80 ton/ day; conversion: 39% for whole whey, 77% for permeate
Gist Brocades (Holland)	Skimmed milk	Yeast enzyme entrapped in triacetate cellulose fibres	Pilot, conversion 71-81% within 20 hrs
Sumitomo (Japan)	Whey and milk	Fungal enzyme covalently bound to ion-exchange resin	Pilot
Amerace Corp. (USA)	Whey permeate	Fungal enzyme covalently bound to silica	Pilot, 90% hydrolysis in 4 min

Source: ref.12,37,72,74,75,80,81

D. Economic estimations

192. Two main methods of lactose hydrolysis exist; these are the acid (catalytic) method and the enzymatic method. The first method is characterized by very severe pH and temperature conditions (pH 1-2, temperature 100-150°C), while the second, as was shown in the preceding paragraphs, is carried out under considerably milder conditions (pH 3.5-8, temperature 5-60°C). The literature contains a comparison of the economics of these two principal whey hydrolysis systems, with the example of the Corning Glass

immobilized enzyme process and the hot acid resin process developed by Permutit (cit. in ref. 72). The study has shown that great difficulties arise in attempting a straight comparison between the two systems. However, both systems appear to be viable if the sucrose price is established at US $ 353/ton (1980).

193. A more recent comparison of the two methods, acid and enzymatic (immobilized), has shown a processing cost of 0.51 Hfl per kg of syrup produced by acid hydrolysis, and 0.73 Hfl per kg of syrup produced by the enzymatic hydrolysis (72). A selling price of 0.92 Hfl can be expected. This analysis has been made based on the following data:

Plant capacity	500 tons of UF-permeate per day
Enzymatic system on	**A.niger** lactase immobilized Duolite ES-762 resin
Production steps for the enzymatic hydrolysis	Electrodialysis, hydrolysis, evaporation
Acid hydrolysis system	Strong acid cation exchange resin, two titanium heat exchangers
Production steps for acid hydrolysis	Reverse osmosis, ion exchange, hydrolysis, evaporation, active-coal treatment

The enzymatic product had 77.5% dry matter, while the acid hydrolysed product had 62.5% dry matter (72).

194. The semi-industrial experiences by Milk Marketing Board (U.K.) and Union Laitierre Normande (France) jointly with Corning Glass Works (USA) in the enzymatic hydrolysis of lactose in various kinds of whey, particularly cheddar sweet whey and casein acid whey (see paragraphs 185-187), led the companies to the following conclusions (80). Under Western European typical conditions, an 80% lactose hydrolysis of permeate can be achieved at a total cost close to 0.5 Fr.Francs per kg of lactose, or 0.02 FF/liter of permeate at 40 g/liter. This cost is valid for a typical 200 m^3/day plant and includes capital depreciation, labour, chemicals, utilities, enzymes, etc. De-ashing of whey was estimated as 0.25 to 0.80 FF/kg lactose (varies according to level and technology used; 50% by electrodialysis would be at 0.25 FF while total de-ashing by ion-exchange would be at 0.80 FF), and concentration was estimated as 0.5 FF/kg lactose. Taking these fractions together, total cost "at the gate of the 200,000 liter/day plant" of an hydrolysed lactose syrup would be 1.25-1.80 FF/kg lactose, which is "quite attractive" (80).

195. Shown below is the equipment cost estimate for Corning's pilot plant designed to process 4.5 tons of deproteinized acid whey per day. Processing cost included labour and supplied cost (as shown below in paragraph 199), capital or equipment costs taken at 20% annually (including depreciation, maintenance, taxes, etc.) and cost of the immobilized lactase. The total cost, though, does not include the cost of de-ashing or concentration and assumes the cost of deproteinized whey to be zero (79).

Equipment cost estimate for Corning's pilot plant for the enzymatic hydrolysis of lactose in deproteinized acid whey (4,500 kg/day lactose, 50% hydrolysis)

Equipment	No.	Plant cost (1974 US $)
Column	1	17,200
Storage tanks (50 m3)	2	36,000
Process tanks		
400 l	1	800
1200 l/agitator	1	5,330
Pumps		
centrifugal (80 l/min)	3	19,200
metering	1	4,200
Heat exchanger	1	7,200
Instruments		16,000
		105,930
Contingency (10%)		10,590
Total		$ 116,520

Source: ref. 79

196. Processing costs in Corning's pilot plant were estimated in the range of $ 22-88 per ton lactose, depending on plant size, cost for immobilized enzyme, and percent hydrolysis. One particular example is shown below.

Operating cost estimate for Corning's pilot plant for the enzymatic hydrolysis of lactose in deproteinized acid whey (4,500 kg/day lactose, 50% hydrolysis)

	Man-hr/day	1974 US $
Labour		
Backflushing	3	
Monitoring	3	
Laboratory	2	
	8x$/4.50/hr	36
Supervisor	3x$ 6.00/hr	18
Overhead and fringes		54
Supplies (acid, etc.)		20
Cooling costs		3
Total		$ 131

Processing cost per kg lactose = 2.9 c ($ 29/ton)

Source: ref. 79

Reducing the salt level by ion exchange or electrodialysis results in projected costs of $ 44-110 per ton, including capital costs comparable to those for the hydrolysis system. Similar additional costs would be encountered for concentrating the product from 5% solids to the 50% or higher solids level necessary for sweetener substitution. Overall cost of the sugar product is estimated, according to (79) to be $ 180-220 per ton. That price looked attractive, according to Corning Glass Works (79), compared to the 1974 price for corn syrups ($ 370/ton, dry basis).

197. For the commercial-scale plant which was announced by Corning Glass Works in 1979, and where deproteinized whey (optimal demineralization) has been continuously treated in a packed-bed reactor followed by vacuum concentration of the hydrolyzed syrup (the product throughput is 200 m^3/day), the estimated costs expressed as cent/kg of hydrolyzed lactose are 9-13 cents for demineralization by ion exchange, 9-11 cents for 80% hydrolysis of lactose, and 7 cents for concentration of syrup to 50-60% solids. A zero volume was placed on the deproteinized whey which may be the permeate from an ultrafiltration system (67).

198. Shown below are data by Lehigh/Connecticut Universities and Lehigh Valley Dairy on process economics based on a projected throughput of 45 tons of whey/day (for the main aspects of this base case see paragraph 178).

Hydrolysis plant equipment cost estimate as projected by Lehigh/Connecticut Universities (45 ton whey/day)

Equipment	No.	Plant cost (1978 US $)
Column	1	18,000
Holding tanks (50 m3)	2	32,000
Process tanks		
400 l	1	800
1200 l/agitator	1	5,200
Pumps		
centrifugal (80 l/min)	2	8,400
metering	1	4,200
Heat exchanger	1	7,200
Instruments		16,000
		91,800
Contingency (10%)		9,200
Total		**$ 101,000**

Source: ref. 75

199. The next table gives operating and fixed costs for a 45 ton whey/day hydrolysis plant. Depreciation and interest are taken as 15% of the capital investment which is equivalent to a straight line depreciation over a 20 yr lifetime, zero salvage value, and a borrowing cost of 10% simple interest per year (75).

Operating and fixed costs for hydrolysis plant (45 ton whey/day) as projected by Lehigh/Connecticut Universities

	1978 US $/day
Operating costs	
cost of immobilized enzyme	248
labour cost	50
supplies (buffer, sanitizing, cleaning agents)	20
overheads and fringes	50
process steam	3
	371
Fixed costs	
depreciation and interest (15% of capital investment)	42
maintenance and repairs (10% of capital investment per year)	28
	70
Total cost of hydrolysis per day	$ 441

Source: ref. 75

200. From the table above, it can be seen that a major component of hydrolysis cost is the price and lifetime of the immobilized lactase which have been assumed to be $ 220/kg and 2 months (75). As the projected cost for the immobilized lactase is estimated to be $ 23/kg for production of the enzyme in large quantity (75), the process will be more profitable in the near future. Besides, even with the current high cost of the enzyme, the overall cost of hydrolysis can be significantly reduced by extending the lifetime of the catalyst. Operating costs and investments for other related unit processes for whey hydrolysis, as well as the daily values which may be realized from whey are summarized in the following table:

Daily operating costs and capital investments, and daily values which may be realized from whey as projected by Lehigh/Connecticut Universities for hydrolysis plant of 45 ton whey/day

	1978 US $
Daily operating cost	
hydrolysis	440
concentration	200
demineralization	100
ultrafiltration	390
	1130

Capital investments	
hydrolysis	101,000
concentration	200,000
demineralization	230,000
ultrafiltration	30,000
Daily values	
sweetness added by hydrolysis	423
protein at $ 2.2/kg	800
waste treatment savings	200
	1423

Source: ref. 75

In this table waste treatment savings means the cost of treating the whey as a waste, a cost that will be saved by utilizing the whey. Then, the hydrolysis of 70% of the lactose in 45 ton/day of whey produces an increase in sweetness equivalent to 897 kg/day of sucrose. If sucrose is valued at $ 0.472/kg, this translates into an added value of $ 423 as shown in the table (75).

201. Shown below are economic estimates available for hydrolysis of lactose in whey on pilot and industrial scale. A straight comparison between the different systems is difficult and practically impossible, especially in the case of the pilot plants because of the different basis used for the calculations, different sizes of plants, etc.

Cost estimates for processing whey by means of immobilized lactase on technological level

Company	Hydrolysis cost, per kg of lactose (dry mass)	Overall cost of kg of the end product (dry mass)	Scale of the processes
Corning Glass Works	9-11 cents (1979)	25-31 (1979)	Industrial
Nutrisearch Co.	11 cents (1984)		Industrial
Corning Glass Works, Milk Marketing Board, Union Laitiere Normande		1.25-1.80 FF (1980)	Semi-industrial
Valio Laboratory	19 cents (1979)		Pilot
Lehigh/Connecticut Universities	8-10 cents (1978)	19.6 cents (1978)	Pilot
Rohm GmbH	4.4-6.7 cents (1983)		Pilot
Corning Glass Works	2.2-8.8 cents (1975)	18-22 cents (1975)	Pilot

Source: ref.67,72,74,75,79,80

202. From the above table it can be concluded that since the cost of corn syrup in Western Europe in the beginning of the 1980's was 44-56 cents per kg, hydrolyzed lactose syrup provides a cheaper alternative. In the U.S. the comparison is not as favourable since corn syrup is lower in cost (22-27 cents per kg); however, there are other considerations which may make the process economically viable. For example, it may still be worthwhile to recover the more valuable whey protein and convert the lactose into hydrolysed lactose syrup than pay whey disposal charges. The continuous success of the Nutrisearch Company, now Nutrisearch International Corporation (see paragraph 205) is an appropriate example in this respect.

E. Scale of the processes

203. Since the early 1980's, the concept of industrial processes for lactose hydrolysis in dairy products by means of immobilized enzymes has evolved into a reality. In paragraph 191 the current state of the technological application of immobilized lactase preparations on pilot and industrial scale is shown. It should be noted here that the development of enzyme engineering, exemplified in the development of immobilized lactase research and applications, reflects generally the basic trend of industrial biotechnology, that is formation of joint venture companies, especially in the United States.

204. In 1982 Corning Glass Works has formed two joint venture companies - in the USA and Western Europe - to make and market hydrolyzed whey sugars and protein. Jointly with Britain's Milk Marketing Board, which operates 22 creameries producing half of all the whey in the U.K. - 680 million liters a year (82) - Corning Glass Works formed a firm named Specialist Dairy Products. Initially, the firm is to build and operate a small unit for market development at the Ashton Creamery (82). Jointly, with the Kroger Company (Cincinnatti, USA), Corning has created Nutrisearch Co., which in 1984 started up a $ 15 million plant in Winchester, Kentucky for processing cottage cheese whey with immobilized lactase (see paragraphs 188-190). The Nutrisearch plant, which is the biggest in the world in the area of enzyme engineering for the dairy industry, converts 400 tons of raw cottage cheese whey per day, or about 130,000 ton/year (81).

205. In March, 1986, it was announced that Eastman Kodak Company (Rochester, New York) has joined the Nutrisearch Co. The new company is being incorporated as Nutrisearch International Corporation with Corning, Kroger, and Kodak as equal partners (83). Currently, Nutrisearch has plants in Winchester (Kentucky), Maelor (France), and in Wales (U.K.) and additional plant locations will be selected to meet future growth needs. Among products currently produced by Nutrisearch are baker's yeast, "cream" yeast for ethanol applications, whey protein concentrates used in egg and milk replacement applications, sweet protein concentrates to replace sweetened condensed milk, and many specialty blends (83).

206. As for the hydrolysis of lactose in milk, the low-lactose milk is being produced in Italy on a small scale and finds a market, particularly among those who are somewhat lactose-intolerant. At the end of the 1970's Centrale del Latte in Milan produced 10 tons of low-lactose milk daily (12, 84). The process relies on the use of sterilized milk and, as a result, the product has a flavour not generally acceptable in some countries in North America and Europe where consumers prefer the taste of milk processed by a milder heat treatment. On the other hand, the product is sweeter and so may appeal to some sections of the population such as children (69).

207. For pilot plants, there are three other processes designed to handle milk: Gist Brocades, Rohm GmbH, and Sumitomo (see paragraph 191). These are continuous processes with short residence times (72).

208. The major industrial application is likely to be in the production of hydrolyzed lactose syrups from deproteinized whey. Such sweet syrups have been used by a number of firms as a source of sugar and, in some cases, as a source of protein in bakery products, in confectionery, in ice cream, feedstuffs, for cattle (instead of molasses), and soft fruit drinks (see paragraphs 151,155,156).

209. Hydrolyzed demineralized lactose syrup is produced by Valio Process in Finland. It contains: glucose, 10%; galactose, 20%; lactose, 10%; and protein plus salts, 10%. It is used at a level of 12.3% in an ice cream mix in order to substitute a part of normal sucrose and milk. Various mix formulations have been tested during ice cream manufacture, and the optimum formula was: 11% fat, 10% milk (80% hydrolyzed), 16% sugars (a part of sucrose has been replaced by corn syrup sweeteners) (72).

210. In several cases, low-lactose milk, obtained by means of immobilized lactase, has been used in order to accelerate the ripening of cheddar cheese with significant cost savings. 80% hydrolysed milk was also used in the manufacture of blue cheese. The product showed improved proteolytic activity. An orange-flavoured beverage prepared from hydrolysed and deproteinized cheese whey was recommended as a shelf-stable drink for athletes (72).

211. According to (29), presumably less than 10,000 tons d.s. lactose hydrolysates was produced in 1984 (which obviously does not include the Nutrisearch process) with the estimated immobilized enzyme amount to be less than 5 ton/year.

VIII. PRODUCTION OF GLUCOSE-FRUCTOSE SYRUPS FROM SACCHAROSE BY IMMOBILIZED INVERTASE

212. The production of high fructose syrups from starch using amylolytic enzymes and glucose isomerase has left a significant impact on the sugar industry (see section I of this review). Similar syrups may be obtained from the hydrolysis of sucrose by means of the enzyme invertase. The sugar mixture produced, containing glucose, fructose, and sucrose, does not show any crystallization problem. The use of immobilized invertase allows its application in a

continuous reactor process. However, as a result of the rapid expansion of the industrial production of HFCS all over the world (section I), interest in the industrial application of invertase has generally diminished. Currently there is only one industrial plant and several pilot plants for the enzymatic hydrolysis of sucrose using immobilized invertase.

A. A background

213. The enzymatic hydrolysis of sucrose proceeds with the formation of equimolecular amounts of glucose and fructose. From an economic point of view, industrial-scale enzymatic hydrolysis must be achieved using a highly concentrated sucrose solution to avoid any dilution and concentration steps (85). The hydrolysis of sucrose can also be accomplished by acidic hydrolysis process, but this method usually has significant disadvantages: colour and by-product formation with a subsequent high cost of refining; a low degree of hydrolysis; and the necessity for using high-purity solutions as a starting material (12). Hence the scaling-up of enzyme engineering processes for sucrose inversion to an industrial scale is still favoured by some experts in the field.

B. Commercial preparations of immobilized invertase

214. Snamprogetti has applied its standard method of immobilization for yeast invertase, which was entrapped in spun fibres of cellulose triacetate (12). For some fibre-entrapped preparations a half-life of 5300 days was calculated after 100 days of use (12). The efficiency of the immobilized preparations decreased by increasing the amount of invertase activity entrapped per gram of cellulose triacetate, indicating a remarkable effect of diffusional limitations.

215. Invertase, which has been used in an industrial packed-bed reactor, was immobilized on granular bone charcoal (86).

216. Immobilization of invertase (Maxinvert 200,000, Rapidase) for continuous sucrose on pilot scale was carried out with 8.8 kg corn grits (Eurama) in a 100-litre stirred reactor. The support particle size was 0.8 mm (specific area of 1 m^2/g).The glucose units of the cellulosic fraction of the support were chemically modified by sodium metaperiodate oxidation to form aldehydes, followed by amination by condensation with ethylene diamine, reduction of the resulting imino bonds to amino bonds with sodium cyanoborohydride, and activation of the amino groups with glutaraldehyde. The immobilized enzyme, when used in a packed bed reactor for continuous sucrose inversion, showed a half-life of 365 days at 40°C with 2M sucrose at pH 4.5, or 90 days at 45-55°C (87).

C. Technological characteristics of processes

217. The continuous hydrolysis of high concentrated sucrose solutions, to produce invert sugar with invertase covalently coupled with corn grits (paragraph 216), was scaled-up to a 17.6 l pilot reactor set in a cane sugar refinery. The reactor was a stainless-steel column 15 cm i.d. and 1 m high, packed with 6 kg of immobilized invertase. The column was temperature controlled with recirculated hot water in the jacket. Industrial sucrose solutions were preheated and pH controlled within a stirred tank before and after hydrolysis. This reactor was fed with highly concentrated sucrose solutions, 65-71% (w/w) at 50-55°C, and it has a productivity equal to 9.1 kg sucrose hydrolyzed/h giving 517 g/l/h, with a 72% conversion rate (85). An optimal working temperature was 55°C. Inversion degrees up to 93% could be obtained with a 65% (w/w) initial sucrose concentration. The performance of the pilot-scale reactor led to the processing of highly concentrated sucrose solutions (about 1 kg/l) from a sugar cane refinery (85).

218. Because of significant inhibition of invertase by the products resulting from sucrose hydrolysis (glucose and fructose), the reactor productivity decreases with decreasing flow rate in the column.

Effect of flow rate on the volumetric productivity of the 17.6 l pilot reactor for sucrose hydrolysis with immobilized invertase (52°C, pH 4.6, a sucrose initial concentration of 69%)

Flow rate (l/h)	Conversion (%)	Productivity (g/l/hr)
5	93.7	278
8	79.2	335
9.5	75.3	335
13.8	72.1	517

Source: ref. 85

219. A small pilot plant for the enzymatic hydrolysis of sucrose has been installed by Snamprogetti (Italy). Yeast invertase entrapped in spun fibres of cellulose triacetate, according to the standard Snamprogetti technology, "showed excellent stability and good efficiency", and its use for the production of invert sugar " compares very well with the traditional processes as far the economic and technical aspects are concerned" (12). A glass column, randomly packed with 90 gm of fibres containing invertase (50 mg protein), was continuously fed a 20% sucrose solution at room temperature; every two weeks the fibres were washed with water-glycerol (50:50, v/v). The invertase fibres were operated under these conditions from 1968 to 1978. During those 10 years there was only a 20% decrease in the initial activity and a total amount of 750 kg of sucrose was hydrolyzed. The possibility of operating at high sucrose concentrations with good activity made it possible to develop an efficient pilot process for completely hydrolyzing 50% (w/w) sucrose solutions at a flow rate of about 6 l./hr/kg fibres, avoiding or reducing the risk of microbial contamination (12).

220. The only industrial application of immobilized invertase was a 44.6 m^3 packed-bed reactor containing granular bone charcoal as a support for the enzyme (cit. in ref. 85). This reactor, fed with a 65% (w/w) sucrose solution at 65°C, showed the volumetric productivity of 11 g sucrose hydrolyzed/l./h giving a 60% conversion rate. This productivity was 40 times lower than the productivity which was obtained at a pilot scale (paragraphs 217, 218). Economic estimates and scale of processes for the enzymatic hydrolysis of sucrose, other than described in this section, were not available to the author.

IX. PRODUCTION OF GLUCOSE FROM STARCH HYDROLYSATES BY IMMOBILIZED GLUCOAMYLASE

221. In the last 30 years, the enzymatic hydrolysis of starch (mostly with soluble alpha-amylase and glucoamylase) to produce glucose syrups has become one of the most important of all industrial processes using enzymes. The main use of glucoamylase is to produce high-dextrose syrups of DE 95 from cornstarch where DE (dextrose equivalent) is a measure of reducing sugar on a dry basis, pure dextrose (glucose) being DE 100 and starch being DE 0 (88). The production of such high-dextrose syrups has grown rapidly, mainly because they are used as the feed for making HFCS (see section I).

222. In producing high-dextrose syrups, cornstarch is liquefied and hydrolyzed to DE 10-15 with alpha-amylase. The resulting dextrin at a concentration of 30-40% is mixed with **A.niger** glucoamylase at pH 4.0-4.5 and held in a stirred tank for 48-72 hr at 60°C. It is essential to reach as high a DE value (and therefore as high a glucose content) as possible. The question of glucose yield in the formation of high-dextrose syrup is of overriding importance, as many of the di- and oligosaccharides in the product have an unacceptable taste, and none is broken down when high fructose syrup is being made (88).

223. The efficient conversion of cornstarch to glucose, by means of immobilized glucoamylase, obviously offers a large industrial advantage. Many studies and developments have been made on it (see ref. 88), but the process has not gone beyond pilot scale plants. Up to the present time, commercial production of glucose from starch by an immobilized catalyst has been unsuccessful.

A. A background

224. General consideration of advances and lessons of enzyme engineering shows that the chance for a successful industrial application of an immobilized enzyme is higher when: (i) the enzyme is expensive, (ii) it is unstable in native form, and (iii) a product of better quality is obtained using the immobilized enzyme instead of the native one. Glucoamylase is none of these. It is relatively inexpensive; it is rather stable in the native form and even worse, immobilization does not generally lead to the stabilization of the enzymes, because the main factor in the stability of glucoamylase in operating conditions is stabilization by substrate (maltodextrines) (89, 90). Immobilized glucoamylase usually gives dextrose syrups of poorer quality, i.e., of lesser DE comparatively with the action of the soluble enzyme.

225. As to the cost, glucoamylase is 25% cheaper than invertase, and 30 times cheaper than lactase (91). In addition, soluble glucoamylase is currently used to process batches of concentrated dextrin solutions at temperature sufficiently high, and pH sufficiently low, to discourage microbial contamination. Therefore, any immobilized form of the enzyme would have to have truly superior qualities compared to with the native enzyme to be adopted for industrial use (88). Unfortunately, there are none; at least they have not been discovered up to now. It can be exemplified as with the thermal stability of the enzyme at the pasteurization temperature. According to (90), immobilized glucoamylase with a half-life of about 3 to 4 weeks at 65°C could be considered a technologically feasible preparation with respect to thermostability. However, in spite of a great number of papers published on immobilization of this enzyme, the half-life of most stable insoluble preparations at 65°C does not exceed 5 to 7 days, and it seems that this value is characteristic of a certain limit of thermostability of glucoamylase (89).

226. Because of the poor thermal stability of commercially available glucoamylase, it is necessary to operate immobilized reactors at about 40°C and to maintain the total system in a sterile condition. One corn sweetener producer, however, shows concern about being able to maintain such conditions in large-scale production (15).

227. Lower maximum yields of glucose, which reduce the quality of high-dextrose syrups with immobilized but not with native glucoamylase, were found, due to slow diffusion of the large substrate molecules into porous carriers, and by slow outward diffusion of the glucose product. Higher concentrations of glucose were therefore found in the pores than in the bulk solution; these higher concentrations led to a greater incidence of reversion reactions, giving, for example, isomaltose and other disaccharides (88, 92).As a result, immobilized glucoamylase struggles to produce a DE product under conditions where a 97 DE product is needed for commercial viability and is actually provided by the native glucoamylase in industrial conditions. Thus, it is the lower operational temperature and potentially lower yield that lead to the chief objections to the use of immobilized glucoamylase.

B. Preparations of immobilized glucoamylase

228. Despite the hurdles indicated above, the immobilization of glucoamylase has been extensively studied. The enzyme has been immobilized onto a multitude of different carriers by entrappment, adsorption, ion exchange, and covalent bonding (88). The greatest number of attempts to immobilize glucoamylase have been by covalent attachment to organic and inorganic carriers. Among these attempts, the most common method is to employ nitrous acid or glutaraldehyde to link the enzyme to amine-activated porous glass or silica.

Immobilized glucoamylase used in pilot plants

Company (Organization)	Immobilization procedure
Corning Glass Works, Iowa State University	Covalent coupling to alkylamide porous silica with glutaraldehyde
Snam Progetti	Entrappment in spun fibres of cellulose triacetate
U.S. Dept. of Agriculture	Adsorption on DEAE-cellulose
University College, London	Covalent coupling with amino-chloro-S-triazyl DEAE cellulose and dichloro-S-triazyl DEAE cellulose
Kyowa Hakko	Covalent coupling with phenol formaldehyde resin with dichloro-S-triazyne
Minmedbioprom (USSR)	Covalent coupling to porous silica with glutaraldehyde

Source: ref. 12,88,92,93

229. The immobilized glucoamylase for use in the pilot plant at Iowa State University (see par. 232) was prepared in place by linking 1.8 kg of **A.niger** glucoamylase (Novo) to 14.3 kg of Corning controlled pore alkylamine silica, previously treated with glutaraldehyde. Approximately 45% of the original activity was retained upon immobilization. The silica-bound enzyme was extremely stable at 40°C, which was the operating temperature of the pilot plant column, and in 30% dextrin solutions, the extrapolated half-life was several years. The measured half-lives ranged from 7.5 hr at 70°C to 519 hr at 55°C (92).

230. Snamprogetti (Italy) has been using glucoamylase (Diazyme^R, Miles Laboratories) entrapped in cellulose triacetate fibers (12). Fibres contained from 90 to 360 mg of Diazyme^R per gram of polymer, and the activity of the immobilized enzyme ranged from 10 to 60% according to the type and the concentration of the substrate. The storage stability of the entrapped enzyme was very good, showing unaltered activity after 6 months at room temperature. With the glucoamylase fibers it was possible to operate the hydrolysis of maltose batchwise for 360 days with little loss of activity (12).

231. It was reported recently that British Charcoals and Macdonalds (Greenock), a Scottish biotechnology company which is part of the Tate & Lyle sugar group, has developed immobilized glucoamylase, the method of the immobilization and the carrier being undisclosed. Using immobilized enzyme, the process is claimed to go to completion in less than 10 min (94).

C. Technological characteristics of the processes

232. The best known was the pilot scale process with a nominal capacity of 450 kg/day glucose operated at Iowa State University in the 70's (92). A flow sheet of that facility is shown in Fig. 15. Starch was slurried in a 1000-litre mix tank with deionized water and its pH adjusted with $Ca(OH)_2$. The slurry was pumped to an open receiving vessel, from which it was fed to a Hydroheater jet, where it came in contact with steam. After a residence time of 3 to 4 min in a coil under pressure, the steam was flashed and condensed, and the cooked starch was collected in a 1000-litre hold tank where it was further cooked in the presence of alpha-amylase for prolonged periods. Following this treatment, the pH of the dextrin was adjusted with concentrated HCl and the fat, gluten, fibre and uncooked or retrograded starch were allowed to separate. The dextrin was then heated to 120°C in a coil with a residence time of 4 min and filtered. The filtered dextrin was pumped to either end of a 1.8 m column of 15 cm diameter, filled to 1.5 m depth with 16 kg of the silica-bound glucoamylase (see par. 229). The residence time, at 450 kg/day glucose production and 30%(w/w) solids content, was 9.0 min, whereas in a reaction with soluble glucoamylase it was 72 hr under similar conditions. The column was mounted on a wheeled base and connected to feed and drain lines with quick-connect fittings, so it could be removed from the system and stored in a cold room between runs (95).

233. The pilot plant runs confirmed that feeds of higher DE led to lower final DE's and glucose concentrations. In all cases higher levels of glucose and DE were obtained when free glucoamylase rather than the immobilized form was employed. Generally, the attained yields were 1-1.5 DE units or 1-1.5% glucose yields, lower than with native glucoamylase under the same conditions. No enzyme deactivation was noted during an 80-day run. When microbial contamination became heavy, it was controlled by washing the column with a saturated aqueous solution of chloroform (92).

234. One of the principal designers of the pilot plant, Dr. Peter J. Reilly, suggests that the two main operational objections to the use of immobilized glucoamylase for glucose production - decreased yield and the possibility of microbial contamination - can be overcome, and as an alternative he suggests very small particles of the enzyme carrier and use of biocidal solutions (88).

235. A pilot plant, which included a 50-litre stirred tank reactor and a column reactor, has been used in the USSR for the hydrolysis of a 25 to 30% maize starch solution preliquefied by acid up to a DE of 35 to 40 (93). The reactors contained glucoamylase immobilized on alkylaminated porous silica. Over "long time" continuous operation, glucose syrups containing 88 to 93% glucose were obtained. Besides, the hydrolysis of 35 to 40% potato starch preliquefied by acid to DE 40 was carried out in a 50-litre immobilized enzyme reactor under batch conditions. As a result, samples of glucose pellets containing 78% dry matter and 60% reducing compounds, were obtained by vacuum concentration of the prepared glucose syrups, and these pellets were used in confectionery applications (93).

236. It was reported recently (96), that the Saccharide Operation Development Association, consisting of eight Mitsui group companies, including Mitsui Co. and Mitsui Sugar, will construct a bioreactor test plant for continuous production of glucose from starch using immobilized saccharifying enzymes with 2 ton/hr production capacity by 1988. Also, its Saccharide Fermentation Laboratory (Kawasaki City) is currently working on producing saccharifying enzymes with high thermal stability.

D. Economic estimates

237. In 1975 Corning Glass Works conducted preliminary studies on the economics of a pilot plant capable of producing 4.5 thousand tons of glucose per year. It was reported that the cost of 1 kg glucose would be between 0.22 and 0.33 cents (97), under the following conditions: (i) using seven columns in sequence, (ii) operational temperature of 50°C, (iii) a 100-day half-life, and (iv) immobilized-enzyme cost of $ 11/kg.

238. In the table below, cost of saccharification by immobilized glucoamylase is shown for a plant capable of producing 45 thousand tons of glucose per year (98). The data were based on experimental observations. The yield of dextrose was estimated as 16.8 kg solids per 1 kg of immobilized glucoamylase per day at 40°C. The immobilized enzyme half-life was estimated to be 13.8 days at 60°C, 51.2 days at 50°C, and 402 days (projected) at 40°C. Labour costs were estimated to be about $ 4.00/m^3 for a plant of this size.

Cost of saccharification by immobilized glucoamylase (IG).
Basis 45,000 ton/yr plant (20% per year plant cost for maintenance, depreciation, interest, taxes, etc.).
The 1974 estimated cost of the soluble enzyme process was $ 17.8/m^3 dry solids

Operating tempera-tures (°C)	enzyme utilization in number of half-lives	Processing cost ($/m^3 dry solids)	
		IG cost ($/kg): 11	44
40	3	11.9	18.3
40	2	11.1	18.5
40	1	11.1	22.3
50	3	13.6	28.0
50	2	13.3	30.1
50	1	15.1	40.2
60	3	23.1	68.1
60	2	24.7	77.1
60	1	32.5	111.4

Source: ref.98

239. It was concluded (98), that since the estimated cost of purified glucoamylase is less than \$ 4.00 per kg of the immobilized enzyme, most of the latter is attributable to carrier and immobilization cost. Besides, it was indicated that at the lower temperatures (40°C and below) immobilized glucoamylase (IG) costs as high as \$ 40/kg could be competitive with the soluble batch process(\$ 17.8/m^3, where \$ 4.4/m^3 enzyme cost, \$ 4.0/m^3 labour cost and \$ 9.4/m^3 capital cost, if installed in a new plant), while at 60°C an unrealistically low IG cost of \$ 6/kg would be necessary to be competitive. It was noted that increased plant size or capacity above 45,000 ton/yr would only slightly decrease costs, primarily through labour cost savings. Estimated column capacities and plant costs for 45,000 ton/yr plants under various operating conditions are shown below.

Plant cost estimates. Basis: 45,000 ton/yr plant, 1974 prices

Operation temperatures (°C)	Enzyme utilization (in number of half-lives)	Column capacity (m^3)	Plant cost (x1000,\$)
40	3	34	800
40	2	27	630
40	1	20	470
50	3	18	660
50	2	14	510
50	1	11	370
60	3	12	580
60	2	10	440
60	1	7	310

Source: adapted from ref. 98

240. Shown below is another comparison of batch (soluble glucoamylase) and continuous (immobilized glucoamylase) processes in terms of the reaction cost (25). As one can see, for the batch process the enzyme is the major cost. For the immobilized-enzyme process, the expensive carrier and reagent are the major cost. Because glucoamylase itself is very inexpensive, the batch process is much less costly than the continuous immobilized-enzyme process. It can be concluded from these data that inexpensive enzymes like glucoamylase require inexpensive carriers (25).

Economic estimates for producing 450 ton/year (dry mass) of dextrose syrup Glucoamylase was bound to agarose by cyanogen bromide. One-year carrier life was assumed

	Batch, soluble enzyme	Continuous, immobilized enzyme
Reaction time (arbitrary set)	20 hr	-
Optimum running time, days	-	70
Annual costs:		
Enzyme	$ 2,400	$ 120
Carrier	-	6,300
Reagent	-	9,800
Reactor shell	600	650
Reaction cost:		
cent per pound	0,30	1,7

Source: ref. 25

241. A comparison of the glucoamylase process with that for glucose isomerase (see Section I) shows that immobilized glucose isomerase made possible the production of an important new product, i.e. HFCS, while the glucoamylase process was aimed at displacing a well-established, highly optimized soluble enzyme system for high volume production of glucose. In the latter situation, system performance and economic must be dramatically superior to merit serious commercial attention. At present, the potential economic benefits of immobilized glucoamylase appear marginal, except for possible capacity expansion or new product applications involving low temperature processing (i.e., light beer) (15).

E. Scale of the processes

242. According to (29), presumably less than 5000 tons of syrup d.s. is produced per year, with the estimated immobilized enzyme amount less than 1 ton/year. The largest producer of the immobilized enzyme is Tate & Lyle, U.K. (29).

X. FUTURE PROSPECTS

243. Some of the future directions of enzyme engineering that appear to have a high potential for industrial developments are listed below, followed by a few comments on each of these directions.

Some future directions of enzyme engineering having high potential for industrial developments

Application of multi-enzyme systems
Enzymatic degradation of polymeric substrates (esp. cellulosic materials)
Bioelectrocatalysis and energy transfer
Enzymatic amplification of weak signals
Enzyme-catalyzed synthesis of fine chemicals
Enzymes in organic solvents, non-miscible with water; enzymes in reverse micelles
Thermostable enzymes; stabilization of enzymes
Recombinant DNA and cell fusion as tools for desiging enzymes and enzyme systems with predetermined technological properties

244. In biological systems, one of the unique consequences of enzyme catalysis is the high degree of specificity and high rate by which sequences of chemical reactions are carried out, with each reaction catalyzed by a different enzyme. Efforts to adapt such multi-enzyme systems for **in vitro** use require considerably more research; but the possibilities merit considerable effort. Work needs to be continued on the stabilization of biologically-produced multi-enzyme systems as found in chloroplasts and liver microsomes for **in vitro** use. At present, the non-biological preparation of multi-enzyme systems is limited to random distribution of different enzymes on the surface of solid supports or in solution (see, for example, 99). An experimental approach for the non-biological preparation of an ordered sequence of different enzymes immobilized on the surface of a solid support would constitute a major breakthrough with great potential for the in vitro application of multi-enzyme systems. Probably, the most advanced group in this direction is one headed by Klaus Mosbach from Lund, Sweden, who has been working with co-immobilization of several enzymes, arranged in an oriented fashion by crosslinking, leading to preparations in which the active sites of the enzymes are juxtaposed to one another (100). Another approach of this group is the fusion of two enzymes by ligating their structural genes using recombinant DNA techniques. It was experimentally demonstrated that the translated gene product was able to catalyze the sequential reaction normally carried out by the separate native enzymes (101).

245. The enzymatic degradation of insoluble polymeric substrates, such as cellulose, lignin, and chitin, continue to present both fundamental and practical challenges for the controlled use of such a process on a large industrial scale. The behaviour of enzymes in the degradation of these substrates is complex, especially since multi-enzyme systems are usually (if not always) involved. Adsorption of these and other surface phenomena as well as the non-regular supramolecular structure of polymers influence the

kinetics of their degradation. Thus, the practical demonstration of economical processes must receive a major input of innovative research. Since cellulose represents replenishable substrate for energy, food, and other necessities of life, there is an urgent push to explore the possibilities for development in **in vitro** processes for the degradation of cellulose (and other polymetric substrates) via enzymatic catalysis (102-105).

246. Bioelectrocatalysis and energy transfer is another direction where our present knowledge of biological energy transfer mechanisms suggests that there may be something unique in in vitro systems with developmental potential. Studies so far have shown that some enzymes can be incorporated into functioning electrodes and can be used thereby to catalyze electrochemical and energy transfer reactions. Various immobilization schemes and electron transfer mediators need to be explored; and the theoretical quantitative description of rates of reaction and rates of energy transfer need to be developed for different electrochemical reactor configurations. The experimental demonstration of enzyme-catalyzed fuel cells, the enzyme-catalyzed biophotolysis of water, the microbial enzymatic conversion of carbohydrates by hydrogen, and the electrosynthesis of energy-rich products are specific examples where a great deal of fundamental as well as practical research is needed. This direction has been studied fruitfully by several groups (106-111). One of these groups was headed by A.Yaropolov at A.N.Bach Institute of Biochemistry, USSR Academy of Sciences, who studied hydrogen electrooxidation and oxygen electroreduction on electrodes with immobilized hydrogenase and lactase respectively. Enzymes immobilized in semi-conducted matrices as a new type of heterogeneous bioanalytical sensor, and new ways for bioelectrosynthesis of organic compounds including complex biological substances (109).

247. The in vitro enzymatic amplification of weak signals is a concept that has its in vivo counterpart in the cascade theory of blood clotting, wherein a very small concentration of clot-promoting tissue factor causes the eventual precipitation of a large quantity of fibrin particles. Both light and ultrasonic signals are suggested as inputs for the enzymatic amplifiers; the former approach has been developed up to the semi-industrial testing of light-sensitive enzyme-containing materials in the USSR (112-115).

248. The enzyme-catalyzed synthesis of fine chemicals is an area in which alternative processes often exist and the selection of commercial processes is highly dependent on processing economics. The synthesis of complex compounds having physiologic or pharmacologic activity, including antibiotics, prostaglandins, or neuropeptides, may be an appropriate avenue for enzyme-based processes; however, each product must be considered separately and in competition with alternative routes of fermentation, non-enzymatic chemical synthesis, or extraction from biological tissue or fluids. The development of economically practical processes for the synthesis or regeneration of high energy phosphate compounds, and the demonstration of ways to couple the hydrolysis of these compounds to the synthesis of organic compounds, could lead to a wider scope of **in vitro** synthesis reactions where enzymatic catalysis might have an advantage (116).

249. In biological systems enzymes catalyze both synthesis and degradation of lipid materials. The mechanistic role of how enzymes function in a lipid environment is not well understood, since many enzymes are assumed to function primarily in an aqueous environment. With lipid, many enzymes may work at the aqueous-lipid interface rather than inside the lipid environment. This topic requires in-depth fundamental research in order to learn how to adapt lipid enzymatic systems for use in vitro. Significant applications in food processing, waste conversion and chemical synthesis can be envisioned (1,117).

250. In water, the equilibrium of many important enzymatic processes is shifted to a great extent towards initial reagents. This shift relates, first of all, to the processes in which the initial reagents are ionized and, therefore, strongly hydrated, and secondly to those in which water is formed as a resultant product (peptide synthesis, etc.). The unfavourable, in terms of the thermodynamics situation, can be improved by performing the enzymatic reaction in the biphase "water/water-immiscible organic solvent" system (118,119). Here the chemical equilibrium can be shifted by lowering the water content in the reaction medium or by choosing an organic solvent which could efficiently extract the product. A micellar medium represents, in fact, a variation of the biphasic water/organic solvent system. Investigations into the problems have attracted a number of research groups, particularly those working in the USA, England, France, Netherland, Japan, USSR (118-136).

251. The scaling-up in the area of enzyme engineering is often hindered because many enzymes even in an optimally immobilized form are not stable enough and more or less rapidly inactivate (denatured). Hence, the problem of enzyme stabilization or reactivation (from the denatured state) is of great importance to enzyme engineering. This is also determined by the fact that in many cases the end product of an enzyme engineering process is subjected to fast microbial degradation (e.g. glucose), and sometimes, the least expensive way to decrease the chance of microbial contamination of an enzyme reactor is to run the reaction process at pasteurization temperature (around 65°C) for a long time, if the process is the continuous one. Very few industrial (and other) enzymes can survive at this temperature for longer than a week (and usually only for several minutes), and this is not enough, as a rule, to protect a continuous enzymatic process from bacterial contamination.

252. On the other hand, there is at present very little nonempirical data on the stability of immobilized enzymes. It will be necessary to establish the molecular basis of stability and inactivation of individual enzymes in both soluble and immobilized forms and then, probably, of multienzyme systems. At the same time (or as a consequence), it is necessary to know how to manipulate the thermostability of individual enzymes on a molecular level and how to reactivate them from their denatured form (at least in specific cases). A major area for future development will be a better

understanding of the denaturation process especially as it is affected by immobilization and by the conditions to which it is subjected in reactors. Two groups working fruitfully in this direction, one in the USA and one in the USSR, should be mentioned (137-141).

253. Considering problems which hamper industrial application of enzyme engineering developments, one rather unexpected problem should be mentioned: sometimes the most suitable enzyme (on the basis of a microbial screening) turns out to be quite unsatisfactory as a result of consequent development and scaling up the enzymatic process. A search for a suitable enzyme preparation for a subsequent industrial application by means of screening of the corresponding strains is directed as a rule to enzyme activity determination, that is, to the amount of the enzyme in a system under study (a culture fluid, a cell lysate and so on). On the other hand, in enzyme engineering processes the quality features of the enzyme are often more important, including thermal stability, sensitivity to product inhibition, catalytic constant, the ability to adsorb onto a solid support, etc. In other words, a highly active culture liquid will not necessarily give a technologically feasible enzyme preparation, because screening tasks and the technology needed often differ in relation to requirements which are addressed in relation to an enzyme (142).

254. There are at least two ways to resolve the general situation: either to look for new natural producers of desired enzymes by means of a "molecular screening", that is to estimate quantitatively specific properties of enzymes (in the course of screening) which are particularly important for the new technology, or to modulate molecular characteristics of the "next generation" enzymes by means of specific changes in a genetic apparatus of strains with the help of genetic or protein engineering. Both ways could lead to discovering or designing enzymes with predetermined technological properties (143).

255. Speaking about the specific changes in genetic apparatus of strains and the improvement of strains in general, it should be mentioned that enzyme engineering should gain in the (near) future from the recent advances of biotechnology/molecular biology: recombinant DNA techniques, gene amplification, and protoplast fusion. The first two approaches utilize the transfer of a gene (or genes) via a plasmid (or other genetic vectors on a molecular level) into another host which becomes a producer of the desired biomolecule; the second approach deals specifically with transfer into a bacterium for amplification of the gene which codes for a useful enzyme. Protoplast fusion is used particularly to improve strains, by combining a high-producing but slow-growing mutant with a poor producing but vigorously growing strain to form a healthy overproducing recombinant strain (144,145).

XI. CONCLUSIONS

256. This analysis of the current state of enzyme engineering at the industrial level may give rise to a number of rather general conclusions and recommendations with emphasis on lessons to be learned. Apparently commercial successes of some enzyme technologies and failure of others are connected more or less directly with the following questions:

(i) How new is the end product which is to be produced by means of immobilized enzymes and what is the demand for it on the market (world-wide or local)?

(ii) What is the cost of the enzyme which is to be used in an immobilized form as the basis of the new technology ?

(iii) What is the cost of the carrier and the immobilization methods?

(iv) Is the heat stability of the immobilized enzyme high enough to run the continuous process at pasteurization temperatures (e.g., around 65°C) to minimize microbial growth problems?

(v) What is the efficiency of the new technology (i.e. degree of conversion into end product) compared with conventional processes based on soluble enzymes or on the use of chemicals?

(vi) What is the quality (i.e. purity, etc.) of the end product obtained by means of the new technology compared with conventional processes?

257. The importance of these questions is particularly relevant when considering the characteristics of glucose isomerase systems that contributed to its commercial success (section I) (13):-

(a) Immobilized glucose isomerase made possible the production of an important new product, HFCS; that is, technology of HFCS production has been developing as a result of the commercial introduction of immobilized enzymes technology. Thus, there is no competition between this process and any conventional technology (chemical, enzymatic, or microbiological).

(b) Glucose isomerase is a rather expensive enzyme, and its multiple use in an immobilized form is especially beneficial even if immobilization costs are taken into consideration. This is the major reason for the commercial success of immobilized glucose isomerase technology as compared with the use of the soluble enzyme.

(c) High heat stability of glucose isomerase has allowed the continuous isomerization process to run at pasteurization temperatures (60-70°C) which is strongly preferred for continuous food processing to minimize microbial contamination problems.

(d) Conversion efficiency is high when immobilized glucose isomerase is used and is equal to the efficiency achieved with soluble enzyme. Conversion efficiency happened to be critical in the immobilized glucoamylase-catalyzed process, where the enzyme at best produces only a 93 DE glucose syrup (see par. 227) instead of the 97 DE product needed for commercial viability; the latter figure can be usually achieved by using soluble glucoamylase.

258. All these conclusions reinforce the importance of economics to the commercial success of enzyme engineering processes. Thus, the economic evaluation of any immobilized enzyme system at a laboratory or pilot scale level is an initial step in process development.

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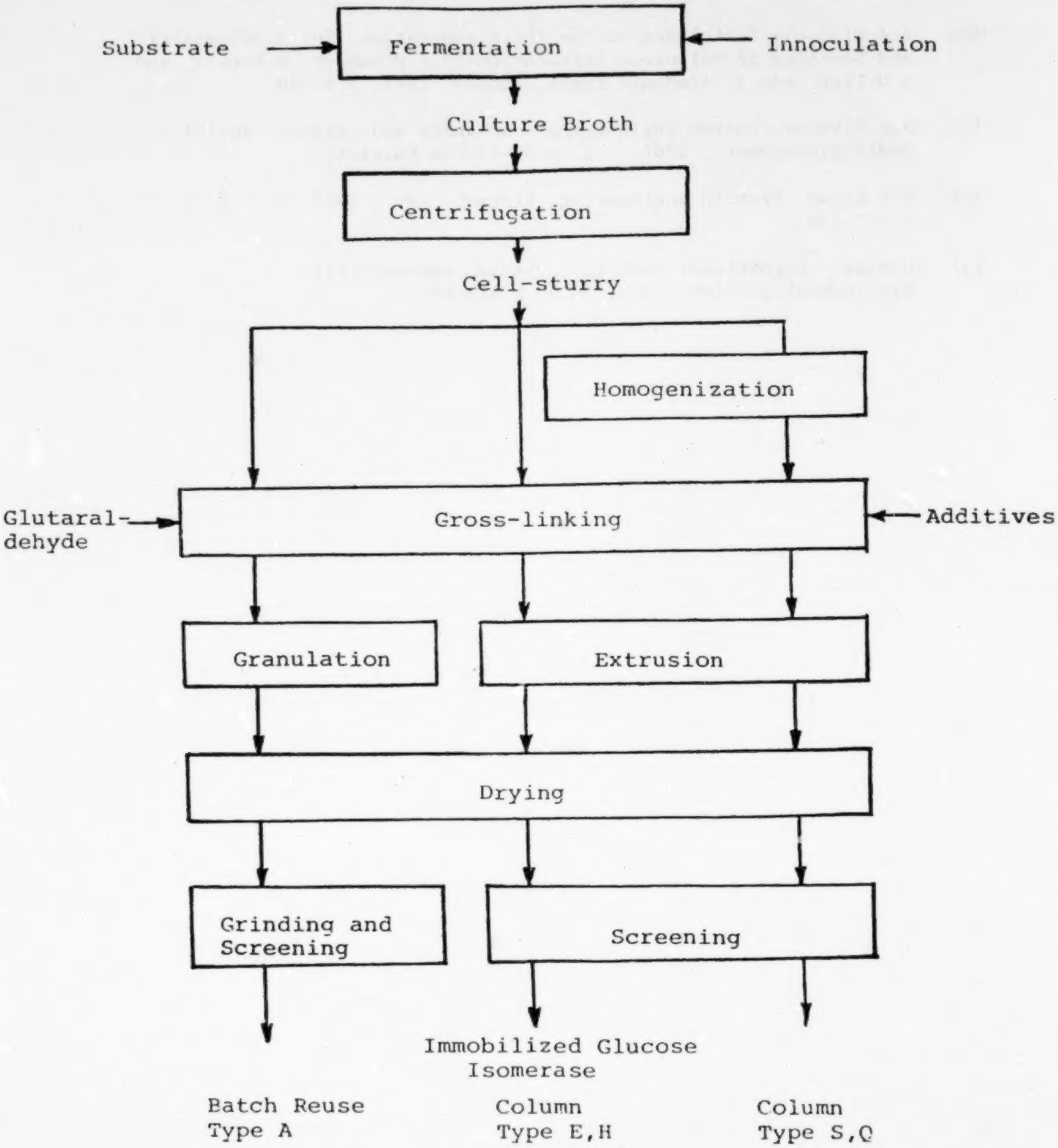
Substrate
Fermentation
Innoculation
Culture Broth
Centrifugation
Cell-sturry
Homogenization
Glutaral-dehyde
Gross-linking
Additives
Granulation
Extrusion
Drying
Grinding and Screening
Screening
Immobilized Glucose Isomerase
Batch Reuse Type A
Column Type E,H
Column Type S,Q

Fig. 1. Steps in the production of immobilized glucose isomerase of "three generations" (Sweetzyme^R, Novo Laboratories Inc.) for three types of reactors (10).

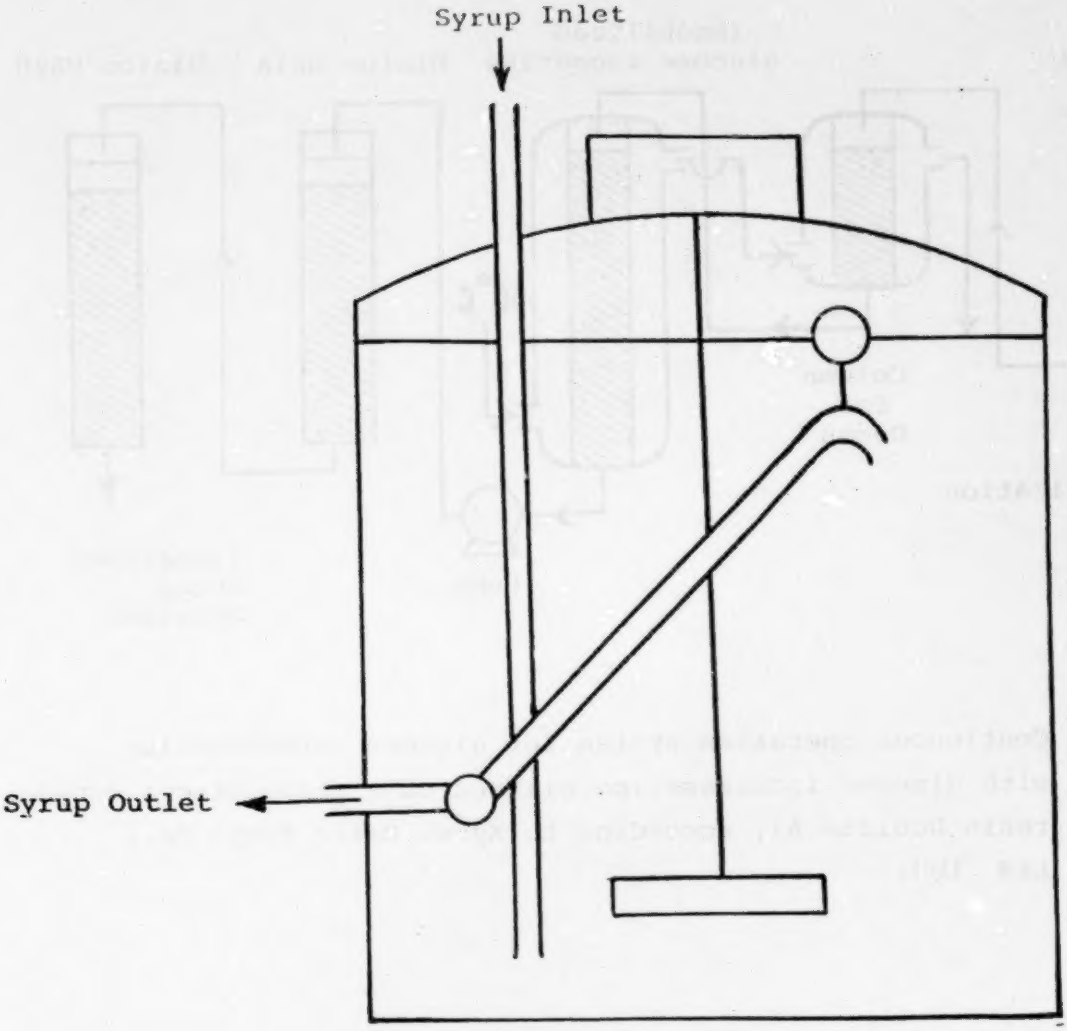
Syrup Inlet
Syrup Outlet

Fig. 2. Batch/reuse reactor for production of high-fructose corn syrup, using powder-type immobilized glucose isomerase of the "first generation" by Novo Laboratories Inc. (10,21).

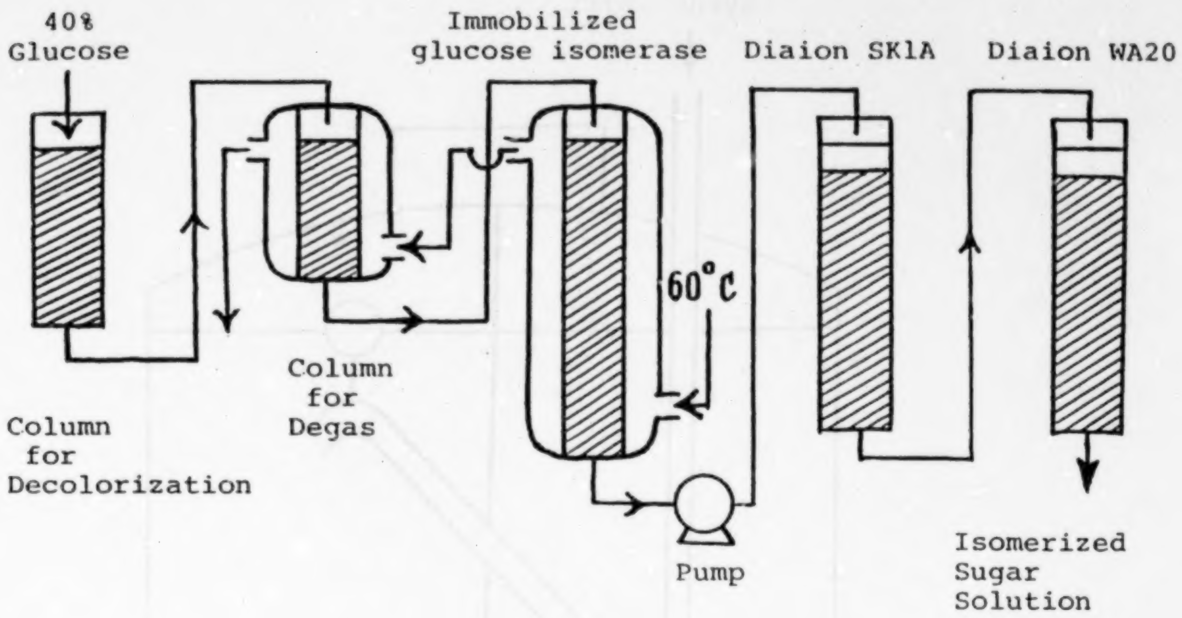
40%
Glucose
Immobilized
glucose isomerase
Diaion SKlA
Diaion WA20
60°C
Column
for
Degas
Column
for
Decolorization
Pump
Isomerized
Sugar
Solution

Fig. 3. Continuous operation system for glucose isomerization with glucose isomerase immobilized on a phenoloformaldehyde resin Duolite A7, according to Kyowa Hakko Kogyo Co., Ltd (16).

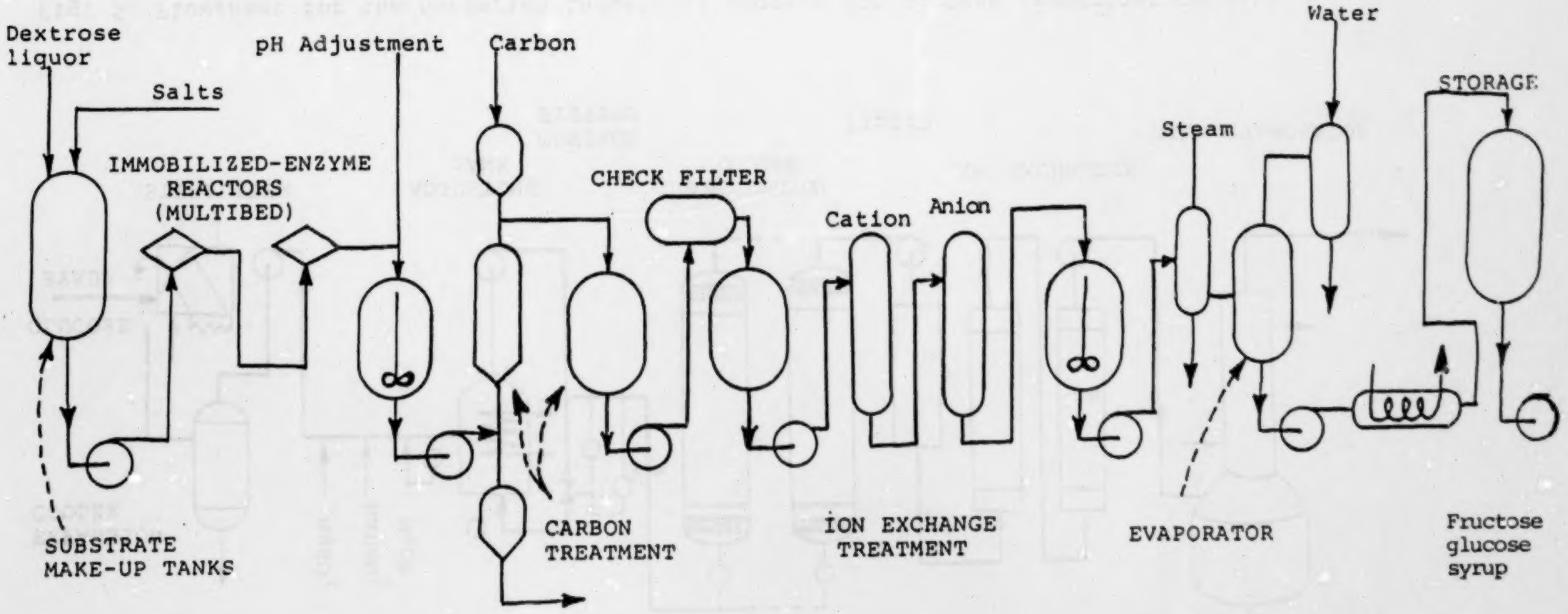
Dextrose liquor
Salts
IMMOBILIZED-ENZYME REACTORS (MULTIBED)
pH Adjustment
Carbon
CHECK FILTER
Cation
Anion
Steam
Water
STORAGE
SUBSTRATE MAKE-UP TANKS
CARBON TREATMENT
ION EXCHANGE TREATMENT
EVAPORATOR
Fructose glucose syrup

Fig. 4. Flowsheet of the technological process for glucose isomerization by Clinton Corn Products Co. (17).

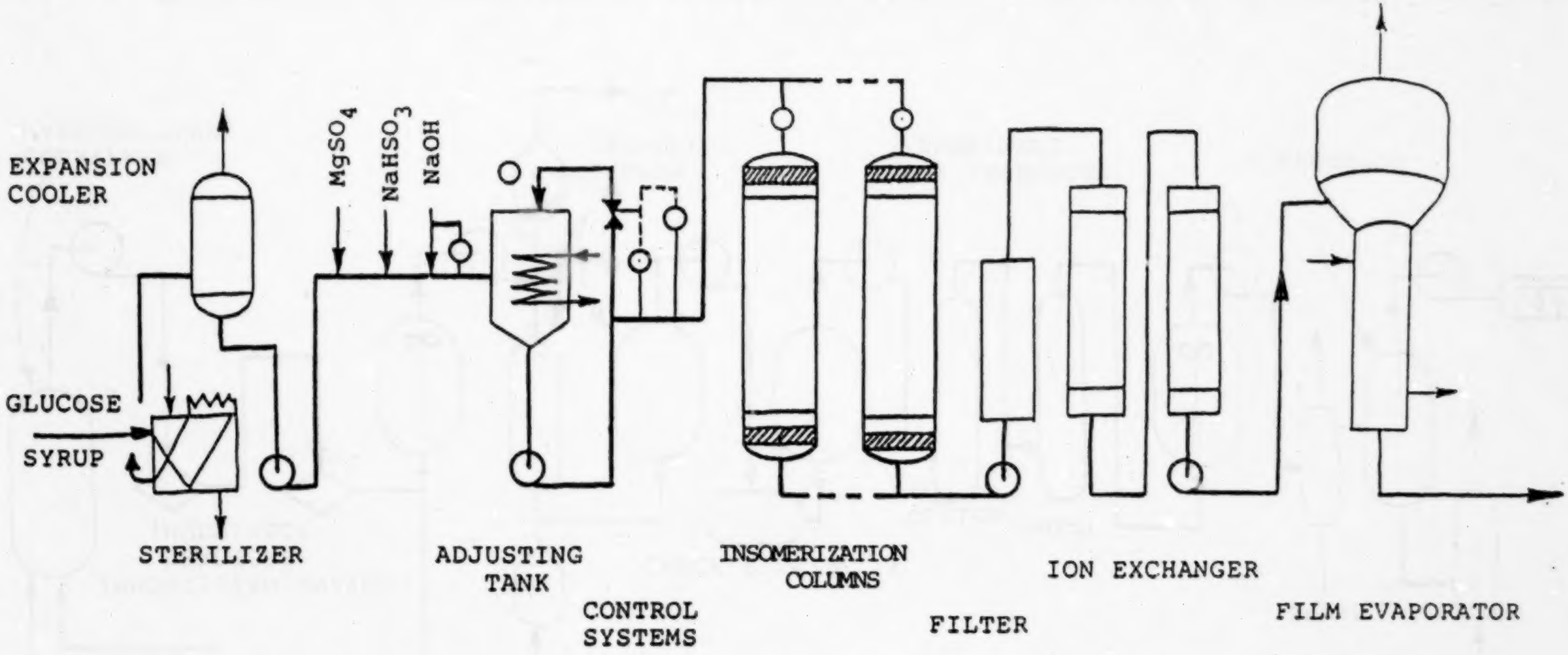
EXPANSION COOLER
GLUCOSE
SYRUP
STERILIZER
$MgSO_4$
$NaHSO_3$
NaOH
ADJUSTING TANK
CONTROL SYSTEMS
INSOMERIZATION COLUMNS
FILTER
ION EXCHANGER
FILM EVAPORATOR

Fig. 5. Flowsheet for the Hungarian industrial process for glucose isomerization with a processing capacity of 400 tons of corn daily (19).

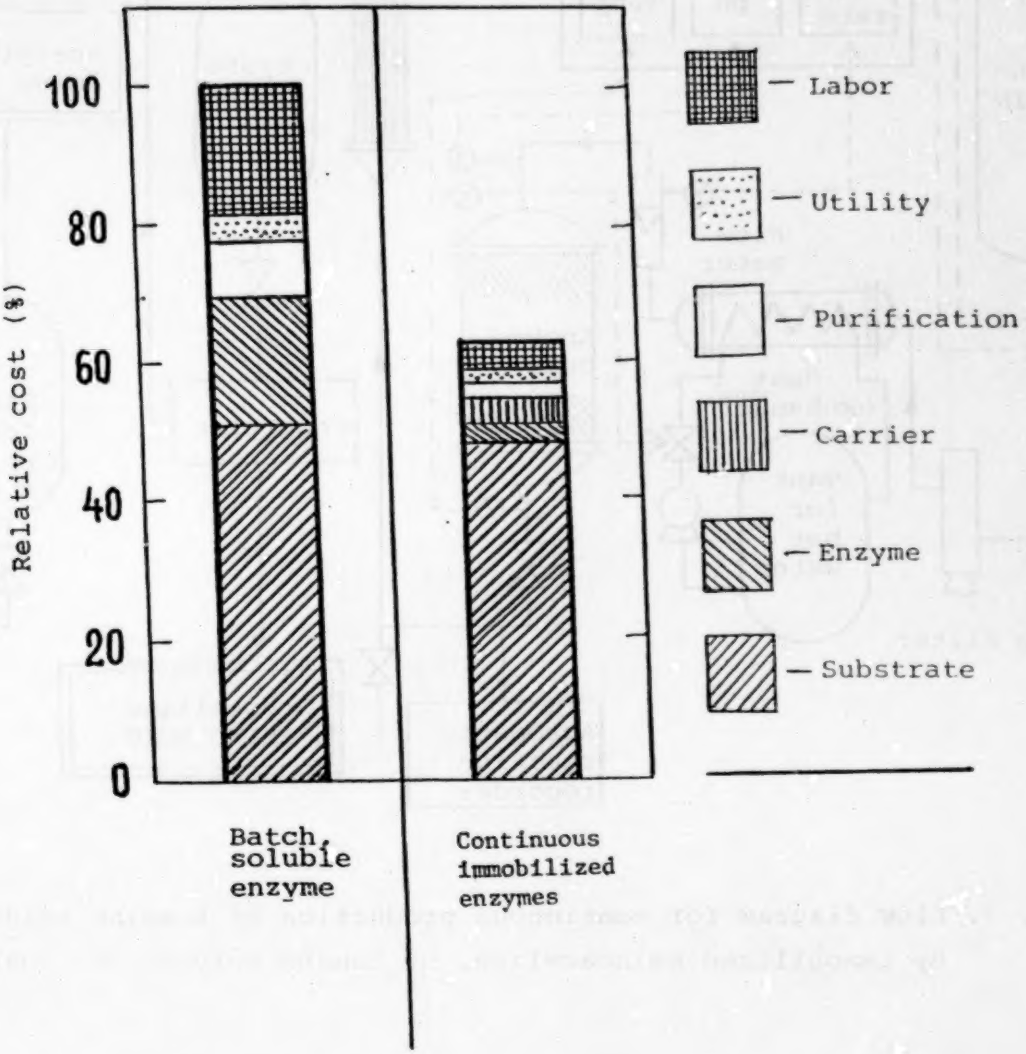

Fig. 6. Comparison of variable cost for isomerization of glucose with glucose isomerase, soluble and immobilized on Duolite A7, by Kyowa Hakko Kogyo CO., Ltd (16).

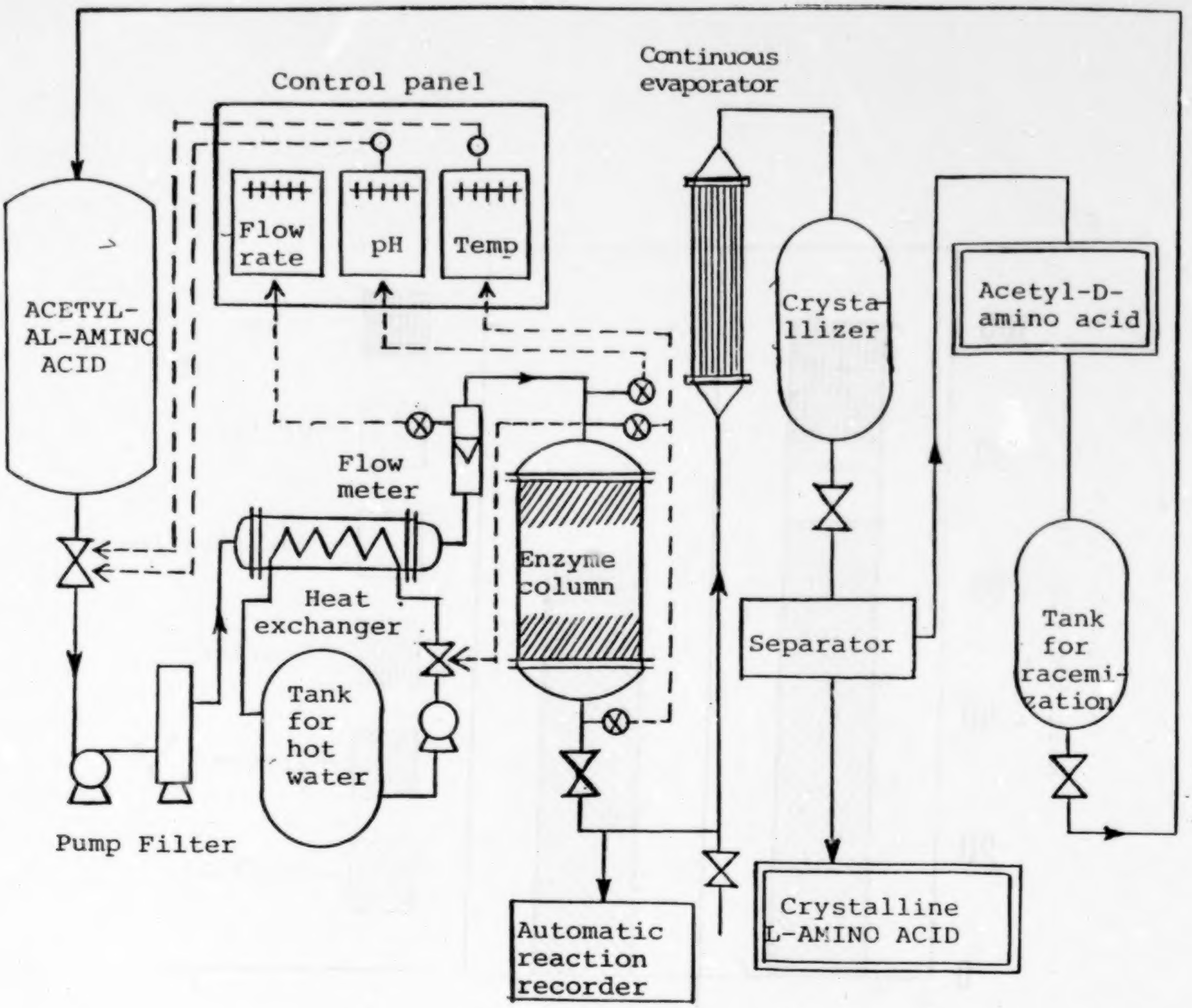
Control panel
Flow rate
pH
Temp
Continuous evaporator
ACETYL-AL-AMINO ACID
Crystallizer
Acetyl-D-amino acid
Flow meter
Heat exchanger
Enzyme column
Tank for hot water
Separator
Tank for racemization
Pump Filter
Automatic reaction recorder
Crystalline L-AMINO ACID

Fig. 7. Flow diagram for continuous production of L-amino acids by immobilized aminoacylase, by Tanabe Seiyaku Co. (38).

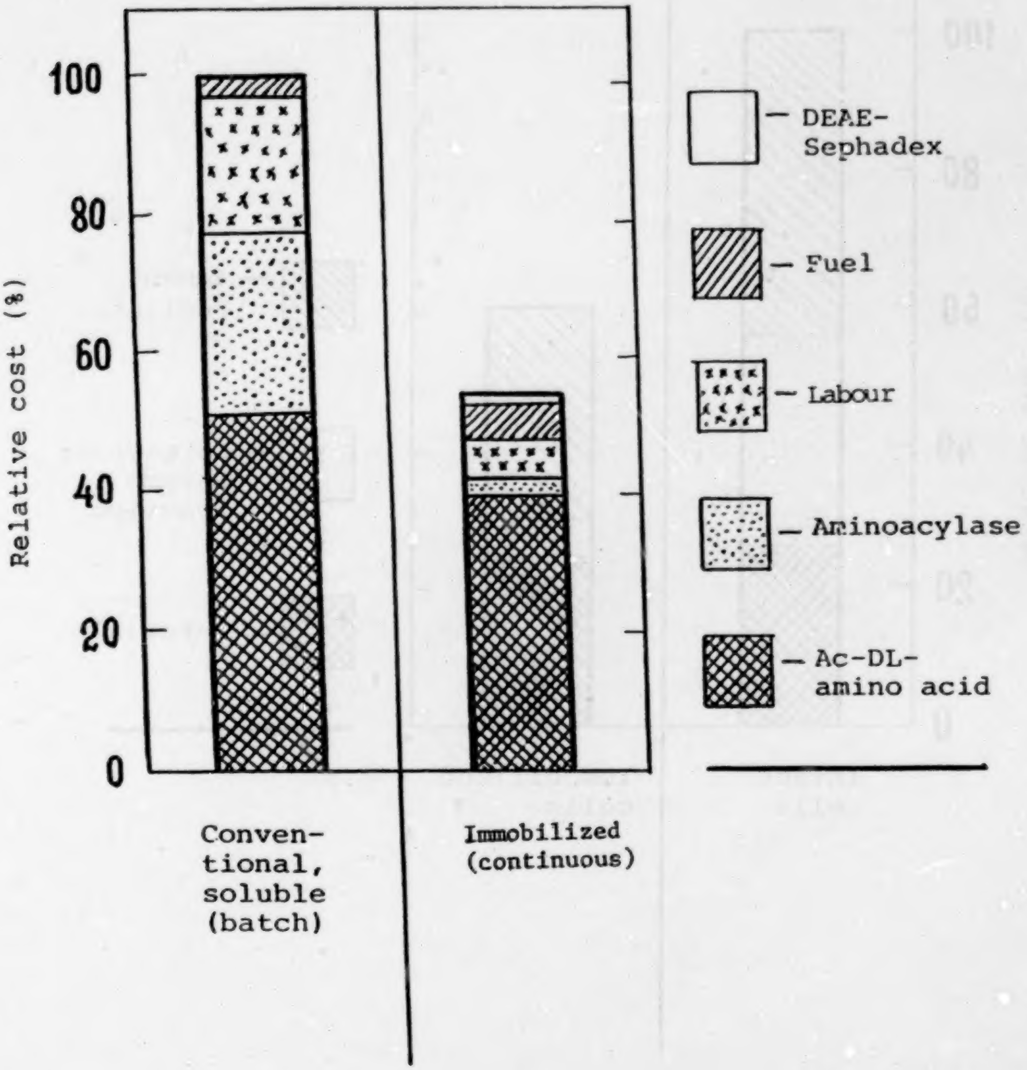

Fig. 8. Comparison of costs for production of L-amino acids with soluble and immobilized aminoacylase, by Tanabe Seiyaku Co. (36).

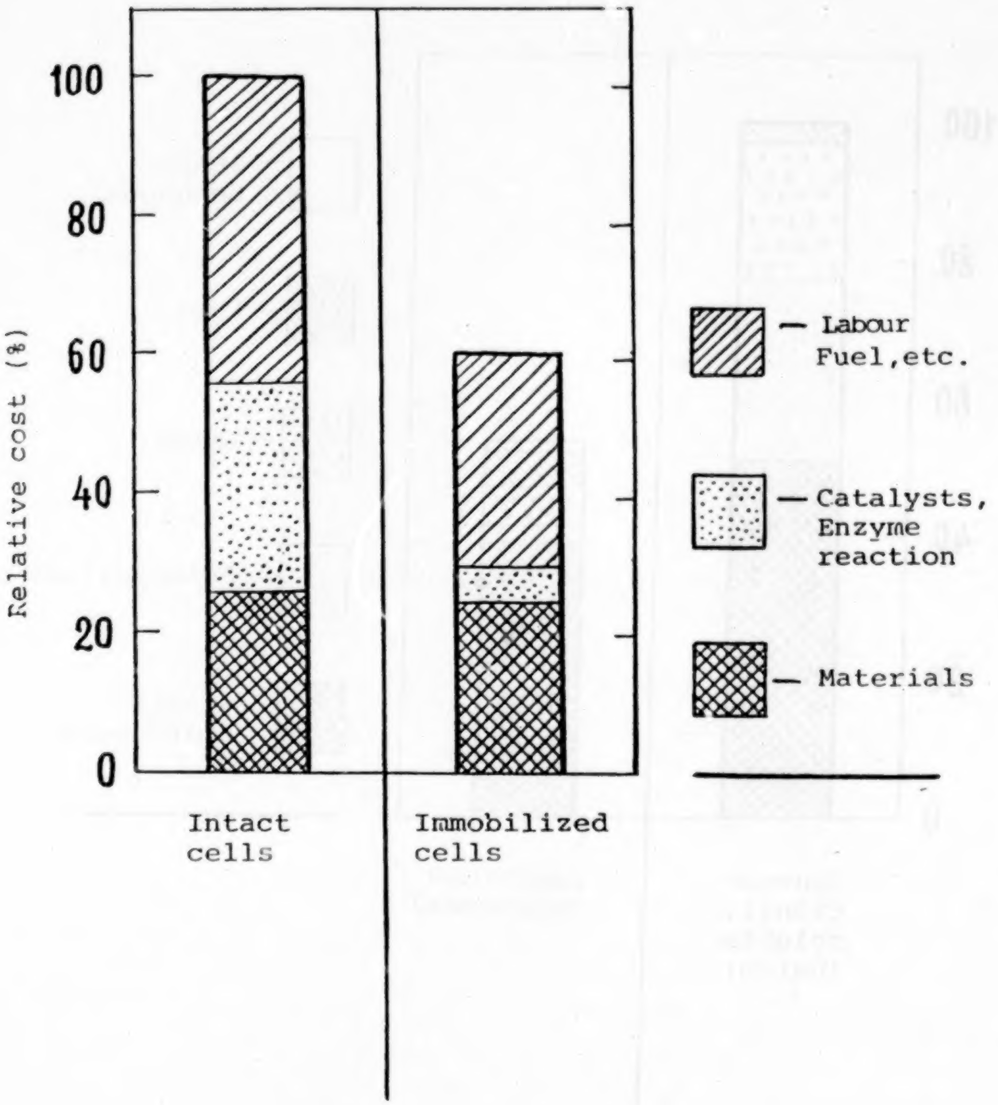

Fig. 9. Comparison of costs for production of L-aspartic acid by the conventional batch process (using intact cells) and the continuous process (using immobilized cells), by Tanabe Seiyaku Co. (38).

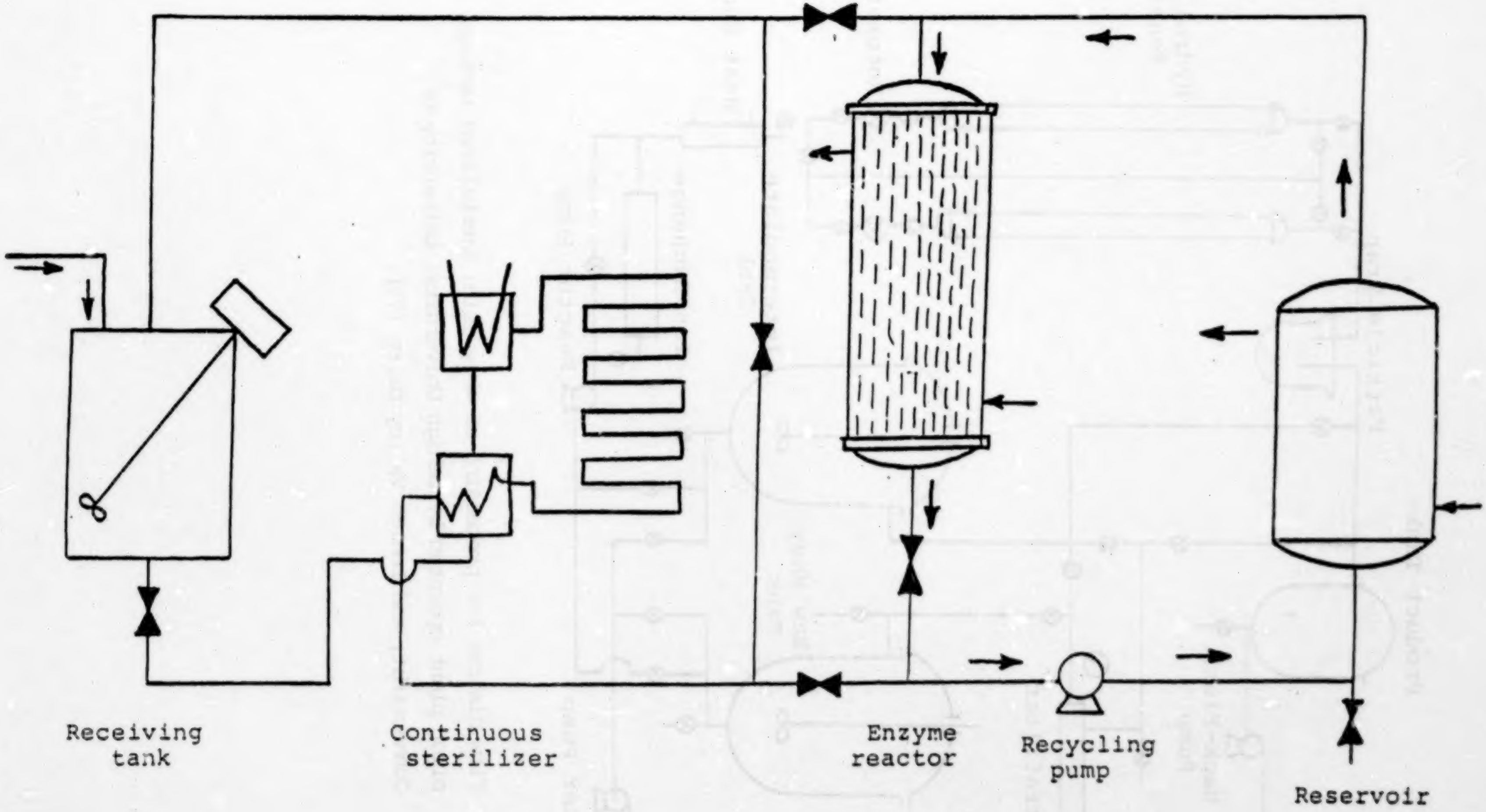
Receiving tank
Continuous sterilizer
Enzyme reactor
Recycling pump
Reservoir

Fig. 10. Flow diagram for continuous treatment of milk with immobilized lactase, pilot plant by Snam Progetti (46).

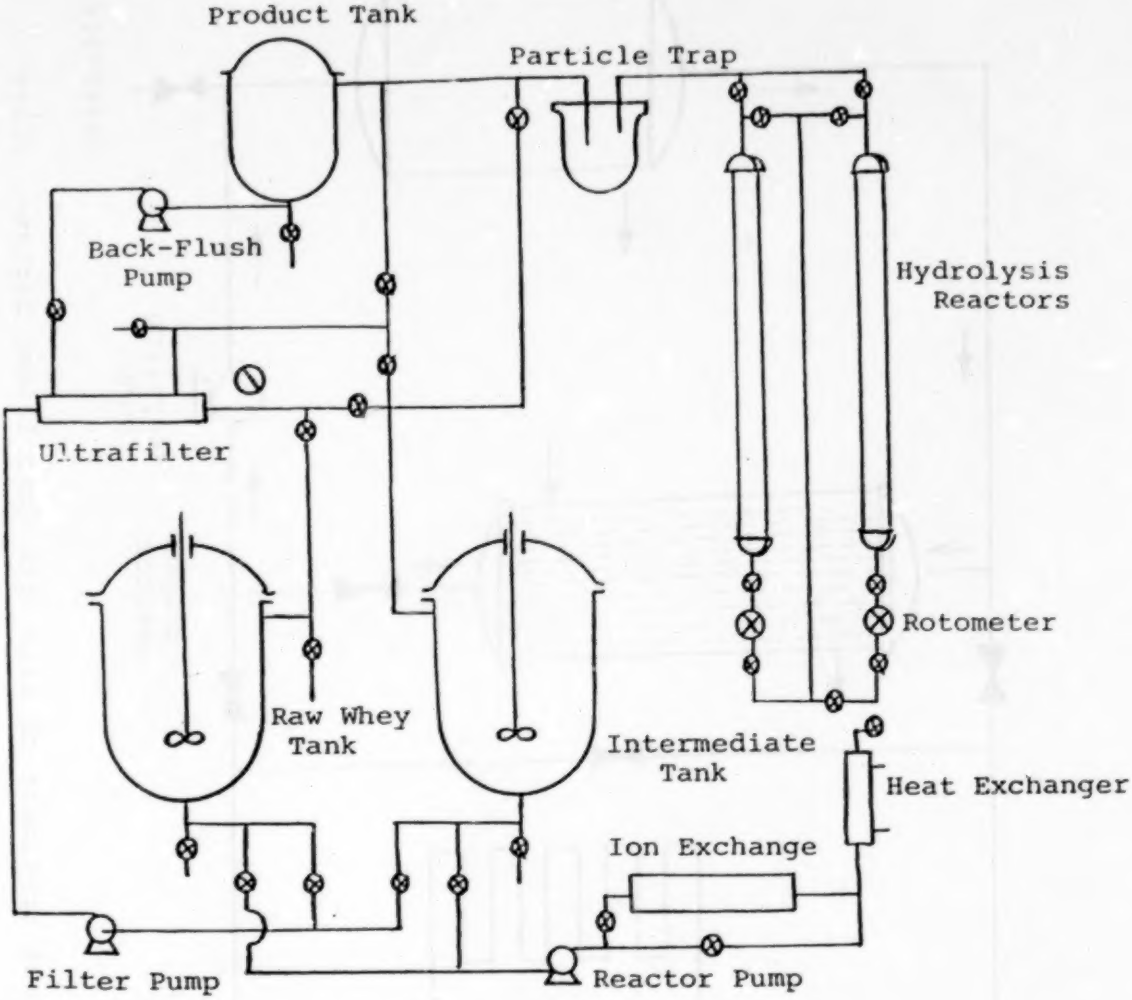
Product Tank
Particle Trap
Back-Flush Pump
Hydrolysis Reactors
Ultrafilter
Rotometer
Raw Whey Tank
Intermediate Tank
Heat Exchanger
Ion Exchange
Filter Pump
Reactor Pump

Fig. 11. Flow diagram for processing of whey using immobilized lactase, pilot plant operated by Lehigh University, University of Connecticut, and Lehigh Valley Dairy (75).

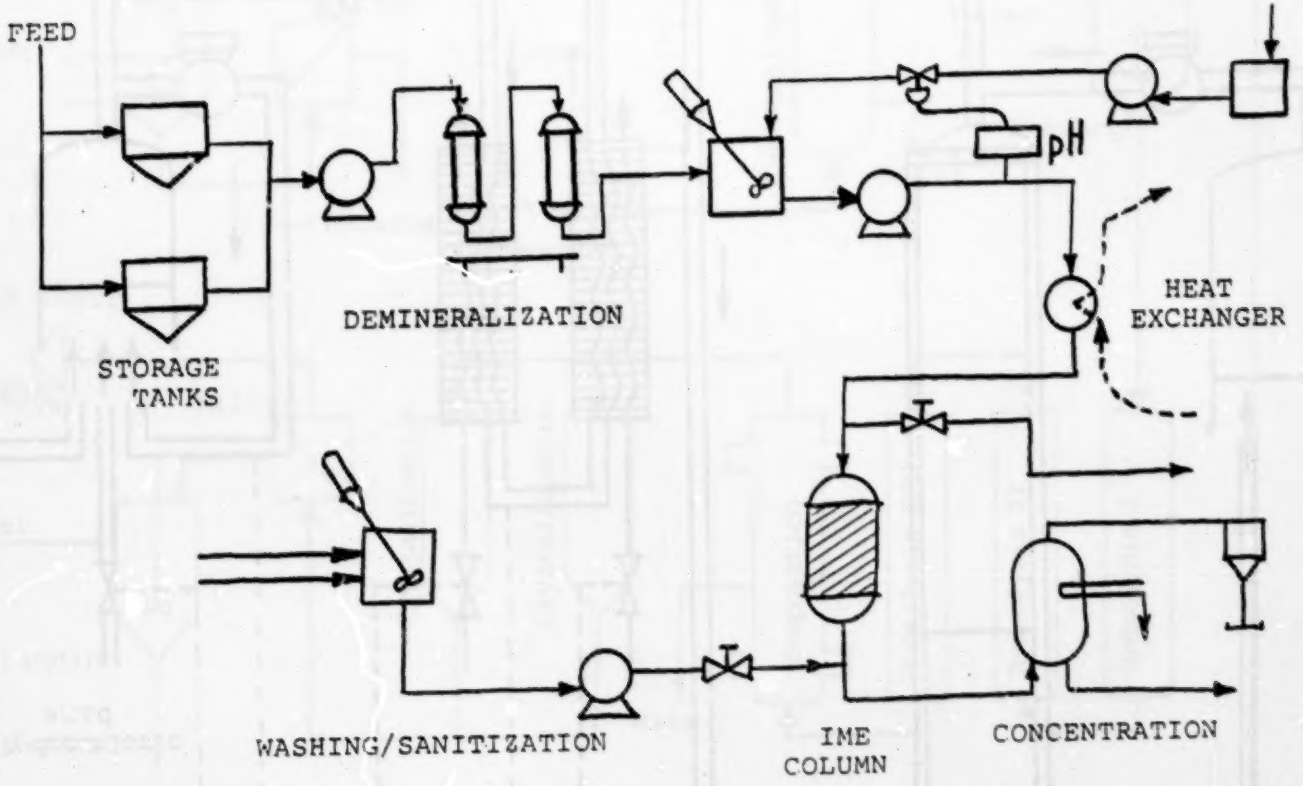
FEED
STORAGE TANKS
DEMINERALIZATION
pH
HEAT EXCHANGER
WASHING/SANITIZATION
IME COLUMN
CONCENTRATION

Fig. 12. Flow diagram for the recovery and hydrolysis of lactose from deproteinized acid whey with immobilized lactase (IME); pilot plant by Corning Glass Works (79).

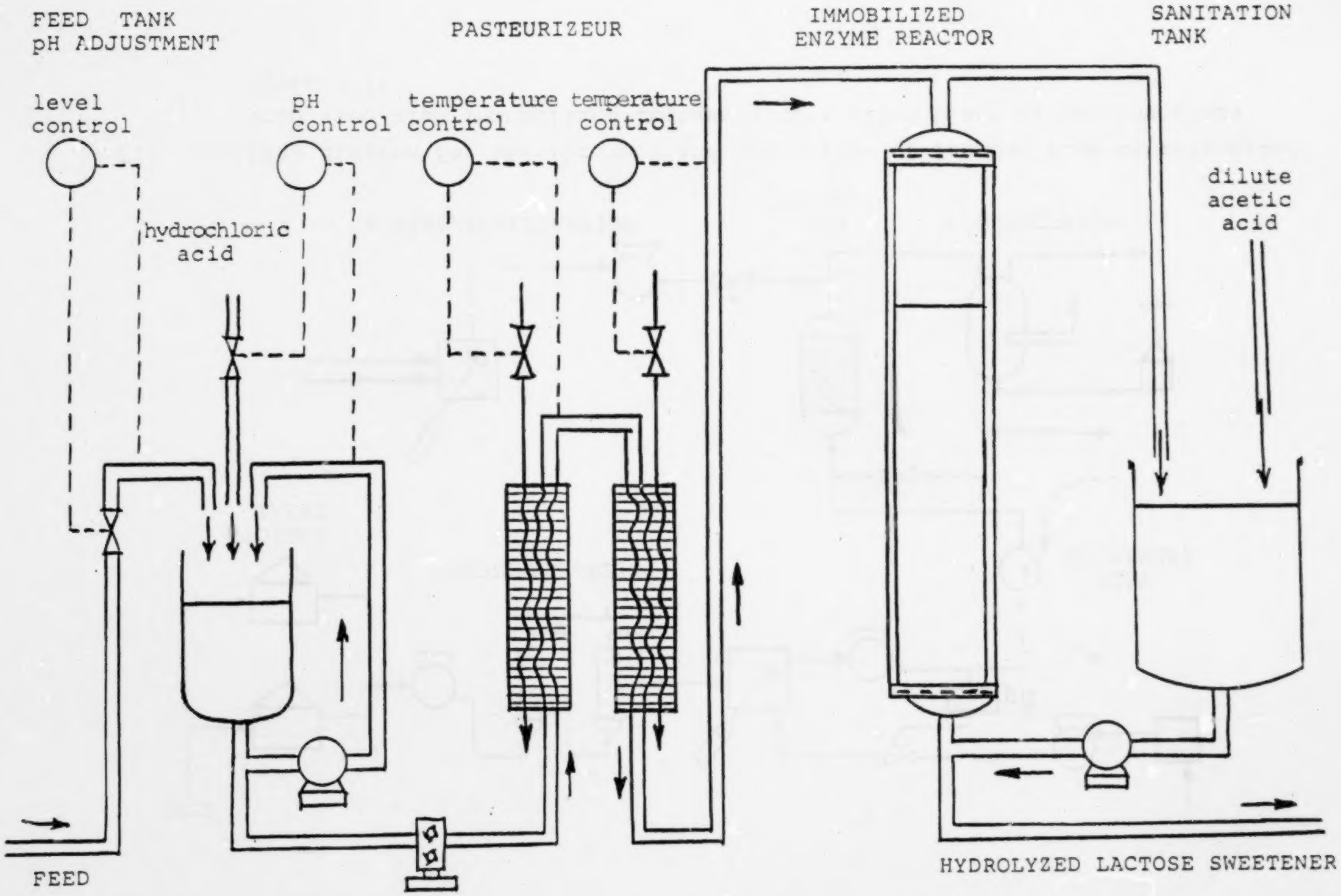
FEED TANK
pH ADJUSTMENT
PASTEURIZEUR
IMMOBILIZED
ENZYME REACTOR
SANITATION
TANK
level
control
pH
control
temperature
control
temperature
control
hydrochloric
acid
dilute
acetic
acid
FEED
HYDROLYZED LACTOSE SWEETENER

Fig. 13. Flow diagram for whey permeate processing steps using immobilized lactase, in a semi-industrial plant operated by Corning, Milk Marketing Board, England and Wales, and Union Laitiere Normande (80).

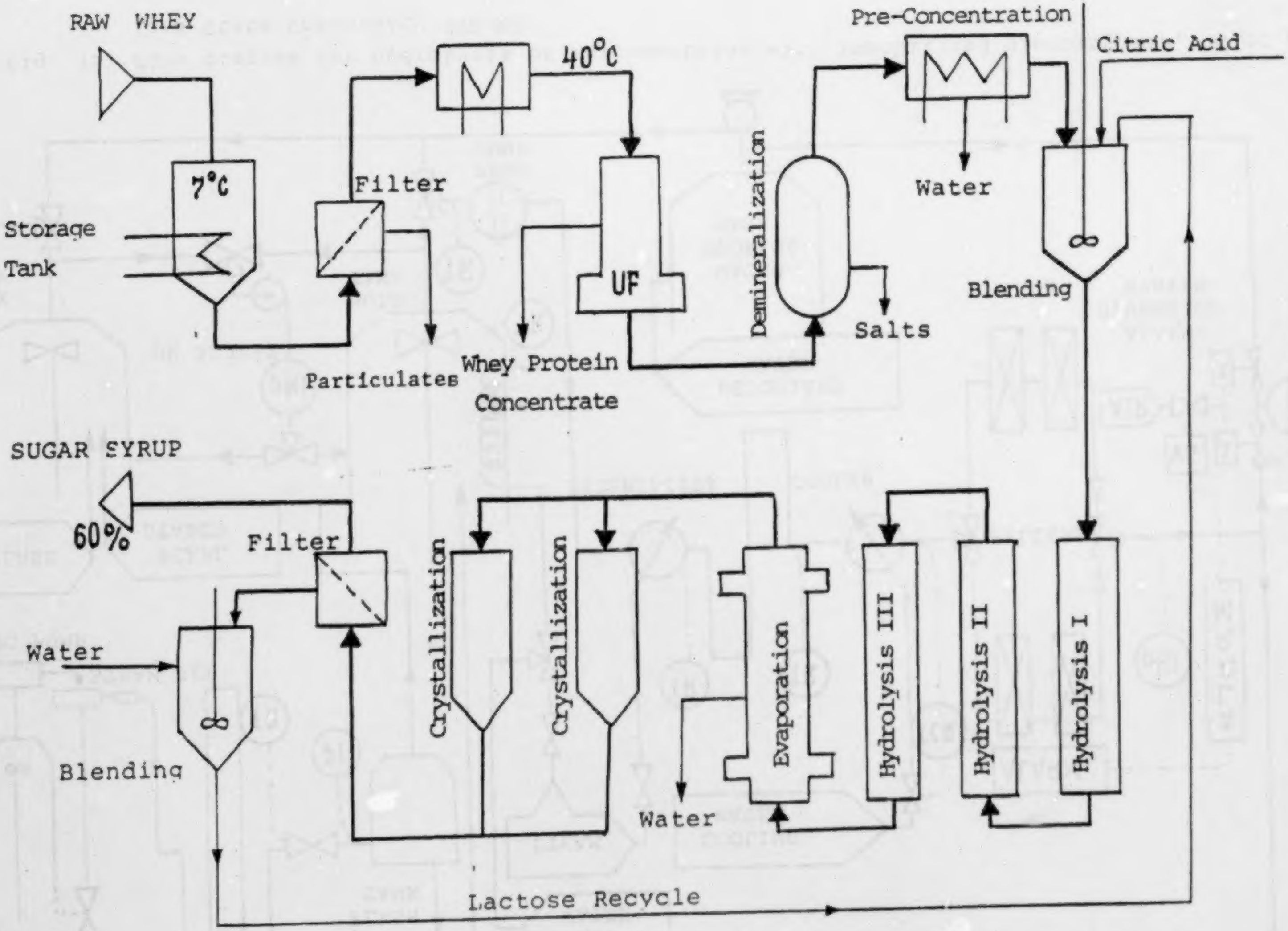
RAW WHEY
7°C
Storage
Tank
Filter
Particulates
40°C
UF
Whey Protein
Concentrate
Demineralization
Salts
Pre-Concentration
Water
Citric Acid
Blending
SUGAR SYRUP
60%
Filter
Water
Blending
Crystallization
Crystallization
Evaporation
Water
Hydrolysis III
Hydrolysis II
Hydrolysis I
Lactose Recycle

Fig. 14. Flow diagram for whey processing steps using immobilized lactase, Pilot plant "Lactohyd", EHT Zentrum, Zurich, Switzerland (68).

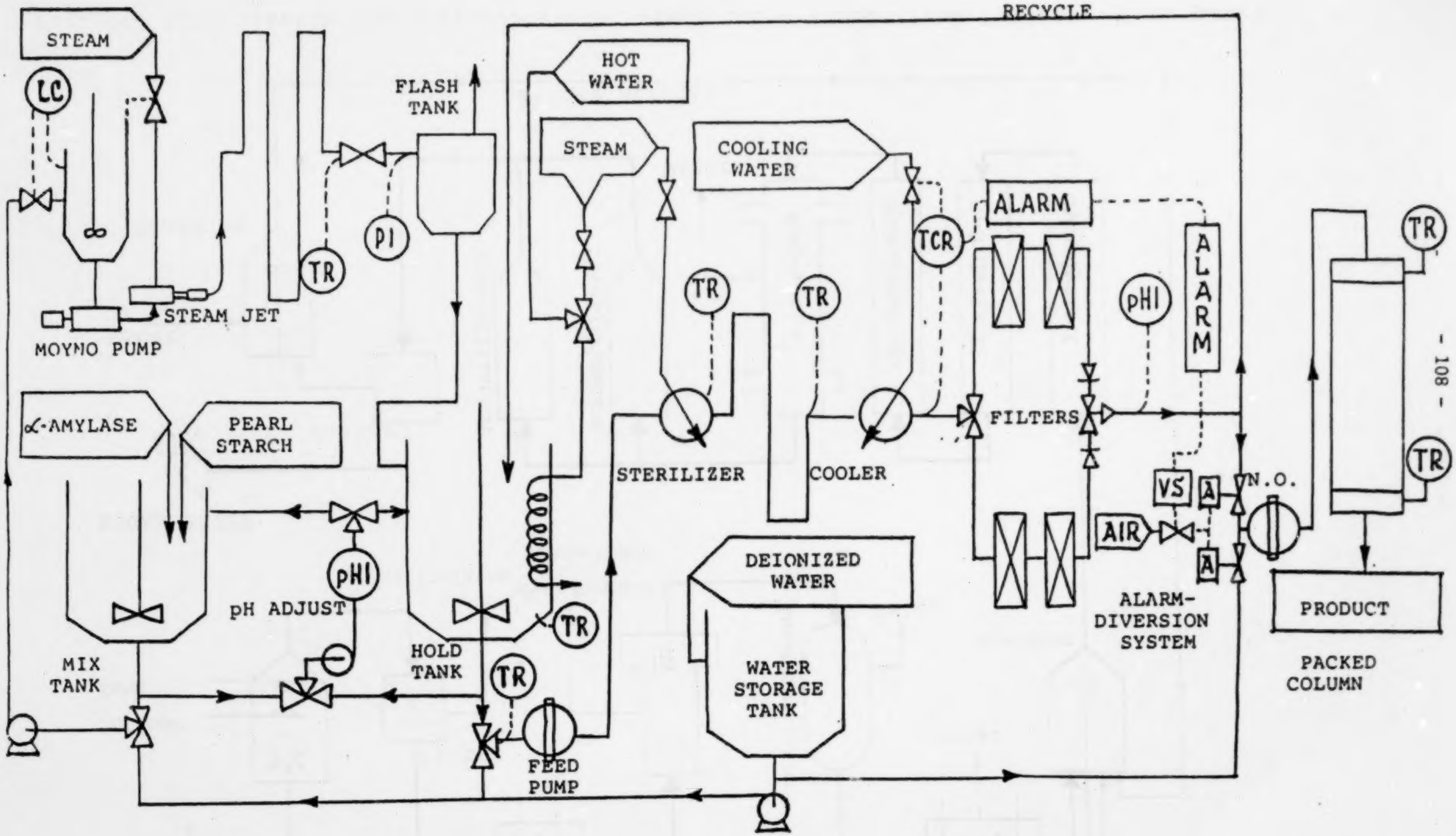
STEAM
LC
MOYNO PUMP
STEAM JET
TR
PI
FLASH TANK
HOT WATER
STEAM
COOLING WATER
RECYCLE
TR
TR
TCR
ALARM
ALARM
pHI
FILTERS
TR
TR
α-AMYLASE
PEARL STARCH
STERILIZER
COOLER
VS
A
A
N.O.
AIR
pHI
pH ADJUST
MIX TANK
HOLD TANK
TR
TR
FEED PUMP
DEIONIZED WATER
WATER STORAGE TANK
ALARM-DIVERSION SYSTEM
PRODUCT
PACKED COLUMN

Fig. 15. Flow diagram for hydrolysis of maltodextrins with immobilized glucoamylase, Pilot plant, Iowa State University (92,95).